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Regulatory role of calmodulin on systems relevant in tumor cells signaling

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Regulatory role of calmodulin on systems relevant in tumor cells signaling

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Licenciada y Máster en Biología Celular, para obtar al grado de Doctor

por la Universidad Autónoma de Madrid

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INSTITUTO DE INVESTIGACIONES BIOMÉDICAS “ALBERTO SOLS”

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Antonio Villalobo, Profesor de Investigación del Consejo Superior de Investigaciones Científicas y Profesor Honorario de la Universidad Autónoma de Madrid, certifica que:

Silviya Raykova Stateva, Licenciada y Máster en Biología Celular por la Universidad "St. Kliment Ohridski" Sofia, Bulgaria, ha realizado bajo mi dirección el trabajo de investigación titulado: **"Regulatory role of calmodulin on systems relevant in tumor cells signaling"** en el Instituto de Investigaciones Biomédicas "Alberto Sols".

Considero que el mencionado trabajo es apto para poder optar al grado de Doctor por la Universidad Autónoma de Madrid.

Y para que así conste a todos los efectos, firmo el presente certificado en Madrid, a 2 de Junio de 2015.

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На майка ми!

To my mother!

“It's deeds we need, not words.”

Vasil Levski

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Както винаги накрая идват тези, които най-много заслужават благодарности и признание. Много често в живота е така, онези, които са те формирали като човек и личност остават в сянка. Тях не ги споменаваме по имена и принос, защото техният принос остава зад завесите, той е незначителен в очите на науката, но всъщност са сърцето и душата ѝ. Това са семейството, приятелите и началните учители. Те са фундаментът и подправката на живота, неговият смисъл, който много често убягва на вглъбената и центрирана в себе си наука, науката, която се съзтезава и надбягва сама със себе си, която както и вселената, се разширява и се опитва да измести всяко кътче от животът ти, понякога центробежно и задушаващо поглъщане, друг път удобно място да избягаш от заобикалящия те свят. Това никога нямаше да е възможно без безпределната помощ на родителите ми Райко и Милка, на сестра ми Златомира, на племенниците ми Радомира и Петя, на бабите и дядовците ми, на цялото ми семейство и приятели, на всичките ми начални, гимназиални и университетски преподаватели. Над всички тях, обаче се извисява тази, която беше и все още е винаги до мен, зад мен, подкрепяща, обичаща, безусловно отдадена и всеодайна- моята Майка. Малко ще е да кажа- Благодаря ти, Мамо...това е за теб!

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1. ABSTRACT/RESUMEN

Calmodulin (CaM) phosphorylated at different serine/threonine and tyrosine residues is known to exert differential regulatory effects on different CaM-binding proteins as compared to non-phosphorylated CaM. In this work we describe the preparation and characterization of a series of phospho-(Y)-mimetic CaM mutants in which either one or the two tyrosine residues present in CaM (Y99 and Y138) were substituted to aspartic acid or glutamic acid. We demonstrated some biological properties of these CaM mutants, such as their differential phosphorylation by the tyrosine kinase c-Src, and their action as compared to wild type CaM, on the activity of two CaM-dependent enzymes: cyclic nucleotide phosphodiesterase 1 (PDE1), and endothelial nitric oxide synthase (eNOS), as well as c-Src and the epidermal growth factor receptor (EGFR). We demonstrated that CaM directly interacts with c-Src in both Ca^{2+}-dependent and Ca^{2+}-independent manners *in vitro*, and that in living cells the CaM antagonist W-7 inhibits this kinase induced by the upstream activation of EGFR, in human carcinoma epidermoide A431 cells, and by hydrogen peroxide-induced oxidative stress, in both A431 cells and human breast adenocarcinoma SK-BR-3 cells. Most relevant and for the first time, we demonstrated that Ca^{2+}-free CaM (apo-CaM) exerts a far higher activatory action than Ca^{2+}/CaM on Src auto-phosphorylation. We also present experimental evidences suggesting that the EGFR from A431 and A549 cells is subjected to *O*-GlcNAcylation, and that CaM may be involved in the regulation of this process through binding to the *O*-GlcNAc transferase (OGT). We detected a positive *O*-GlcNAcylation signal in immunoprecipitated EGFR using immunoblot and two distinct specific anti-*O*-GlcNAc antibodies. Conversely, the presence of EGFR was detected by immunoblot among the *O*-GlcNAcylated proteins immunoprecipitated with an anti-*O*-GlcNAc antibody. These signals were enhanced when a highly specific *O*-linked β-N-acetylglucosaminidase (OGA) inhibitor was present. Most significantly, we detected a positive *O*-GlcNAcylation signal in immunoprecipitated and *N*-deglycosylated EGFR using peptide-*N*-glycosidase F (PNGase F), and from tunicamycin-treated cells when were metabolically labeled with GlcNAz. We also performed *O*-GlcNAcylation assay *in vitro* using immunoprecipitated EGFR and OGT, which resulted in the enhancement of the EGFR *O*-GlcNAcylation signal. We concluded that the EGFR from A431 and A549 tumor cells is subjected to *O*-GlcNAcylation. Furthermore, we present preliminary data using *in silico* studies, combined with binding assays such as Ca^{2+}-dependent CaM-affinity chromatography and co-immunoprecipitation experiments, showing that OGT is a putative CaM-binding protein.

La calmodulina (CaM) fosforilada en diferentes residuos de serina/treonina y tirosina ejerce efectos reguladores diferenciales en una diversidad de proteínas de unión a CaM en comparación con CaM no fosforilada. En esta Tesis se describe la preparación y caracterización de una serie de mutantes fosfo-(Y)-miméticos de la CaM en el que uno o los dos residuos de tirosina presentes en la CaM (Y99 y Y138) fueron sustituidos por ácido aspártico o ácido glutámico. Hemos demostrado algunas propiedades biológicas de estos mutantes de la CaM, tales como su fosforilación diferencial por la tirosina quinasa c-Src y su acción, en comparación con CaM tipo salvaje, sobre la actividad de varios enzimas CaM-dependientes: la fosfodiesterasa de nucleótidos cíclicos 1 (PDE1), la óxido nítrico sintasa endotelial (eNOS), c-Src y el receptor del factor de crecimiento epidérmico (EGFR). Hemos demostrado que la CaM interactúa directamente con Src de forma dependiente e independiente de Ca^{2+} *in vitro*, y que en células vivas el antagonista de CaM W-7 inhibe esta quinasa inducida por la activación aguas arriba del EGFR, en células A431 de carcinoma epidermoide human, y por estrés oxidativo inducido por peróxido de hidrógeno, tanto en células A431 como en células de adenocarcinoma de mama humano SK-BR-3. De mayor relevencia y por primera vez, hemos demostrado que la CaM libre de Ca^{2+} (apo-CaM) ejerce una acción activadora mucho mayor que el complejo Ca^{2+}/CaM sobre la auto-fosforilación de Src. También presentamos evidencias experimentales que sugieren que el EGFR de células A431 yA549 está sometido a *O*-GlcNAcylatión, y que la CaM puede estar implicada en la regulación de este proceso mediante su unión a la *O*-GlcNAc transferasa (OGT). Hemos detectado una señal positiva de *O*-GlcNAcylación en el EGFR inmunoprecipitado usando inmunoblot y dos anticuerpos específicos anti-*O*-GlcNAc distintos. Adicionalmente, la presencia de EGFR se detectó por inmunoblot entre las proteínas *O*-GlcNAcyladas e inmnoprecipitadas con un anticuerpo anti-*O*-GlcNAc. Estas señales se incrementaron cuando un inhibidor altamente específico de la *O*-ligado β-N-acetilglucosaminidasa (OGA) estuvo presente. Más significativamente, se detectó una señal de *O*-GlcNAcylación positiva en el EGFR inmunoprecipitado y *N*-deglicosilado usando péptido-*N*-glicosidasa F (PNGasa F), y a partir de células tratadas con tunicamicina cuando se marcaron metabólicamente con azido-GlcNAc. También se realizaron ensayos de O-GlcNAcylación *in vitro* utilizando EGFR y OGT inmunoprecipitados, lo que resultó en el incremento de la señal de *O*-GlcNAcylación en el EGFR. Llegamos a la conclusión de que el EGFR de las células tumorales A431 y A549 están sometidos a *O*-GlcNAcylación. Además, se presentan datos preliminares de estudios *in silico*, así como ensayos experimentales de unión, tales como cromatografía de afinidad de CaM dependiente de Ca^{2+} y co-inmunoprecupitación, demostrando que la enzima OGT es putativamente una proteína de unión a CaM.

2. ABBREVIATIONS & ACRONYMS

ABA: abscisic acid

AREG: amphiregulin

Apo-CaM: apocalmodulin

Akt: v-Akt murine thymoma viral oncogene homologue

BCA: bicinchoninic acid

BSA: bovine serum albumin

BTC: betacellulin

CaM: calmodulin

CaM-BD: CaM-binding domain

CaM-BP: CaM-binding protein

CaMK: CaM-dependent kinase

CaM KMT: Calmodulin-lysine N-methyltransferase

CDK: cyclin-dependent kinase

CID: collision-induced dissociation

DTT: dithiothreitol

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGFR: EGF receptor

EGTA: ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

eNOS: endothelial nitric oxide synthase

EPR: epiregulin

ErbB: erythroblastic leukemia viral oncogene homologue

ERK: extracellular signal-regulated kinase

ESI: electrospray ionization

ETD: electron-transfer dissociation

EV: empty vector

FBS: fetal bovine serum

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GFP: green fluorescent protein

Grb7: growth factor receptor-bound protein 7

HB-EGF: heparin-binding EGF-like growth factor

HEK: human embryonic kidney

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER: human EGFR receptor

HRGβ1: heregulin β1

IGFR: insulin-like growth factor receptor

IGF-1R: insulin-like growth factor 1 receptor

IRS1: insulin receptor substrate 1

IRS2: insulin receptor substrate 2

IgG: immunoglobulin G

JD: juxtamembrane domain

JAK: Janus kinase

MAPK: mitogen-activated protein kinase

MEK: mitogen-activated protein kinase kinase

MLCK: myosin light chain kinase

MS: mass spectrometry

NLS: nuclear localization sequence/signal

NP-40: nonidet p-40

NT: non-transfected

OGA: *O*-linked β-*N*-acetylglucosaminidase

OGT: *O*-linked β-*N*-acetylglucosamine transferase

ORF: open reading frame

PBS: phosphate buffered saline

PDE: phosphodiesterase

PI3K: phosphatidylinositol 3-kinase

PLCγ1: phospholipase Cγ1

PMSF: phenylmethylsulfonyl fluoride

PNA: peanut agglutinin

PTB: phosphotyrosine binding

PTM: posttranslational modification

PVDF: polyvinylidene fluoride

RAF: v-Raf murine sarcoma viral oncogene homologue

Ras: rat sarcoma viral oncogene homologue

SDS: sodium dodecyl sulphate

SDS-PAGE: polyacrylamide gel electrophoresis in the presence of SDS

SH2: Src homology 2

SH3: Src homology 3

SHP-2: SH2 protein tyrosine-phosphatase

c-Src: proto-oncogene tyrosine-protein kinase cellular Src

SRY: sex determining region Y

STAT: signal transducer and activator of transcription

TK: tyrosine kinase

TCA: trichloroacetic acid

TRIS: tris(hydroxymethyl)aminomethane

v-Src: tyrosine-protein kinase viral transforming protein Src

W-7: *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

W-12: *N*-(4-aminobutyl)-2-naphthalenesulfonamide

W-13: N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide

WGA: wheat germ agglutinin

3. INTRODUCTION

Posttranslational modifications (PTMs) of the side chains of amino acids in proteins are playing pivotal roles in cell signaling. They are orchestrating the protein behavior, activity and interactions. Currently over 200 modifications has been described in the literature such as phosphorylation, acetylation, methylation, glycosylation just to mention a few (Walsh et al., 2005, Jensen, 2006). PTMs exhibit high diversity in terms of structure, stoichiometry, size, complexity and cellular localization (Garavelli, 2004, Walsh et al., 2005). Among the different PTMs, phosphorylation is one of the best characterized and consists of the addition of a phosphoryl group at serine, threonine, tyrosine and histidine residues of target proteins (Fischer, 2013, Puttick et al., 2008). The fact that 500 proteins encoded by the human genome are kinases is highlighting the importance of this PTM (Gomase and Tagore, 2008). Phosphorylation regulates almost all aspects of cell life; it is the driving force in transmitting the extracellular signals to the cell interior, and participates in protein degradation, protein interactions, and gene expression among many others (Fischer, 2013). Of central interest in this Thesis is to study the role of tyrosine (Tyr, Y) phosphorylation of CaM and how phosphorylation affects the functions exerted by this protein, and the role of CaM regulating c-Src and EGFR activation. In addition, other aspects of this Thesis will be to outline the possible *O*-GlcNAcylation on the EGFR, as it has been proposed to potentially occur at its CaM-binding site (Kaleem et al., 2009), and the interaction of CaM with OGT, the enzyme catalyzing the *O*-GlcNAcylation process.

3.1 Ca^{2+} as a messenger

Calcium ion (Ca^{2+}) is one of the major messengers in cell signaling playing pivotal roles in many cellular functions such as muscle contraction, synaptic transmission, gene transcription, cell proliferation, autophagy and apoptosis among many others (Clapham, 2007). In basal conditions free Ca^{2+} is at low concentrations within the cytosol (from 20 to 50 nM) and about 1 mM in the extracellular space (Barritt, 1999). Upon activating the cell by a variety of stimulus the cytosolic concentration of free Ca^{2+} increases to µM levels, and this “jump” in Ca^{2+} concentration makes of Ca^{2+} a very powerful secondary messenger. Several transport systems located at the plasma membrane, endoplasmic reticulum, mitochondria, and other intracellular organelles are responsible for this regulation, increasing or decreasing the cytosolic concentration of free Ca^{2+} such as $Na^{+}(H^{+})/Ca^{2+}$ exchangers, Ca^{2+} channels, and different Ca^{2+}-ATPases (Clapham, 2007, Barritt, 1999, Berridge et al., 2003).

3.2 The Ca^{2+} sensor protein CaM

3.2.1 Historical overview

In 1967 Cheung reported the presence of an activator of brain cyclic nucleotide phosphodiesterase (PDE) and that during purification it dissociates from the enzyme (Cheung, 1967). At the same time Kakiuchi and Yamazaki reported that a heat-stable protein factor along with Ca^{2+} was necessary for the activation of PDE (Kakiuchi and Yamazaki, 1970). Shortly after their discoveries these studies merged and gave birth to a new universe in cell signaling: they have found that this protein was responsible for the activation of PDE in a Ca^{2+}-dependent manner and in the next few years this crystallized in a few papers related to the newly found Ca^{2+} sensor protein that was named calmodulin (Teo and Wang, 1973, Wolff and Brostrom, 1974, Cheung et al., 1975, Cheung, 1980, Klee and Vanaman, 1982). Since then CaM has been isolated from many organisms and it is present in all eukaryotic cell types (Manalan and Klee, 1984, Smith et al., 1987). CaM is also a very conserved protein among different species. For example, there are just a few differences between mammalian and *Drosophila melanogaster* CaM, where only three amino acids are substituted at positions 99, 143 and 147 (Smith et al., 1987).

3.2.2 Structure of CaM

Since the discovery of CaM a large number of structural studies has accumulated. CaM is an acidic single polypeptide protein that contains 148 amino acid residues and has a molecular mass of 16.7 kDa (Means and Dedman, 1980). Its structure consists of two terminal globular domains connected by a flexible central linker. Each globular domain has two Ca^{2+}-binding sites known as EF hands (Means and Dedman, 1980, Chin and Means, 2000). When Ca^{2+} binds to apo-CaM (Ca^{2+}- free state) a transition occurs to the holo-state (Ca^{2+}/CaM complex), which causes the exposure of large hydrophobic patches of the protein (Figures 1A and 1B). The exposure of these hydrophobic patches is the critical event that drives CaM to bind its targets, as it is shown in Figure 1C representing a CaM-myosin light chain kinase (MLCK) peptide complex (Chin and Means, 2000). These hydrophobic patches are rich in methionine residues: which stabilize the open conformation of Ca^{2+}-loaded CaM and helps it in recognizing many different target proteins with diverse amino acid side chains (Bhattacharya et al., 2004). The different EF-hands have different affinity for its ligand; the EF hands at the C-terminal have a high Ca^{2+} affinity (K_d ~10^{-6}, M), which is 10-fold higher than the N-terminal EF hands affinity for Ca^{2+} (K_d ~10^{-5}, M) (Bhattacharya et al., 2004). Therefore, a different Ca^{2+} affinity of the four EF-hands allows CaM to adopt

many conformations and to be saturated to various degrees (Chin and Means, 2000, Bhattacharya et al., 2004). For example, for the activation of CaMK-II only two Ca^{2+} ions bound to the C-terminal sites are required (Shifman et al., 2006) while four Ca^{2+} are required for the activation of the EGF receptor (Dagher et al., 2011). Thus, the degree of saturation is the fundamental property that makes it dynamic and flexible, and also allows it to bind to several hundred different partners (Shifman et al., 2006, Crivici and Ikura, 1995).

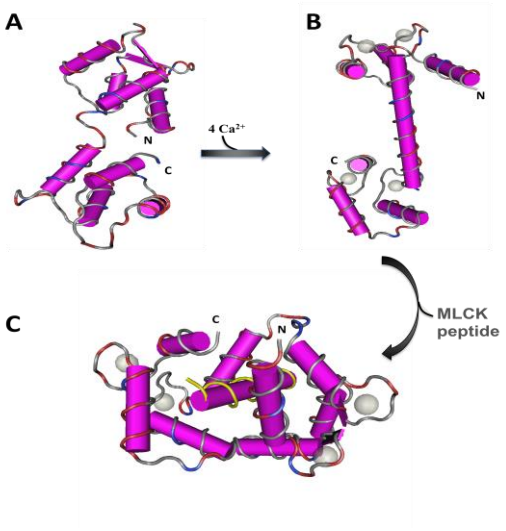

Figure 1: Three-dimensional structures of CaM in its different conformations: (A) Ca^{2+}-free CaM (pdb code: 1CFD); (B) CaM loaded with Ca^{2+} (pdb code: 1CLL) and (C) Ensemble of structures (PDB code: 2K0F) representing a CaM-myosin light chain kinase (MLCK) peptide complex (yellow). Ca^{2+} ions are shown in grey. The structures were drawn using the Cn3D program. The N-terminal (N) and the C-terminal (C) of the protein are also shown.

3.2.3 CaM binding domains

Based on the location of the hydrophobic groups, which serve as anchor points, CaM binding motifs have been classified in the following categories: 1–5–10, 1–8–14, 1–16, IQ, and the IQ-like motifs (Table 1) (Bhattacharya et al., 2004, Jurado et al., 1999). The key interaction occurs through hydrophobic residues such as Phe (F), Trp (W), Ile (I), Leu (L), or Val (V) and typically the C-terminal of CaM interacts with the first amino acids and the N-terminal with the hydrophobic residues at positions 10 and 14. In the case of 1-16 motifs the N-terminal of CaM binds the amino acid at first position and the C-terminal to the residue in position 16 (Bhattacharya et al., 2004). Although Ca^{2+} binding is necessary for CaM to bind to most of its target proteins, apo-CaM is also able to bind and regulate many proteins such as neurogranin, neuromodulin, IRS-1, myosin, and p68 helicase among others (Jurado et al., 1999, Wang et al., 2013).

CaM-BD	Motif sequence
1-5-10	(FILVW)xxxx(FAILVW)xxxx(FILVW)
1-8-14	(FILVW)xxxxxx(FAILVW)xxxxx(FILVW)
1-16	(FILVW)xxxxxxxxxxxxxx(FILVW)
IQ	(FILV)Qxxx(RK)Gxxx(RK)xx(FILVWY)
IQ-like	(FILV)Qxxx(RK)xxxxxxxx

Table 1: CaM-binding domains. The different CaM-binding domains (CaM-BD) including the IQ and IQ-like motifs sequences are shown.

CaM: functional implications

Since CaM is regulating a large number of proteins it is not of surprise that this Ca^{2+} sensor is involved in the regulation of many cellular processes, such as cell proliferation, apoptosis, muscle contraction, reproductive processes, cell motility, cytoskeleton architecture and function, autophagy, gene expression, osmotic control, ions transport, and protein folding among many others (Chin and Means, 2000, Jurado et al., 1999, Berchtold and Villalobo, 2014).

3.2.4 Posttranslational modifications of CaM

The primary function of CaM is to transduce Ca^{2+} signaling in cells by binding hundreds of proteins including many kinases, it is not of surprise that CaM also undergoes posttranslational modifications. Up today CaM has been found to be subjected to acetylation, methylation, phosphorylation and glycation.

3.2.4.1 Acetylation

Watterson *et al.* in 1980 shortly after the discovery of CaM reported that the N-terminus of CaM undergoes acetylation (Watterson et al., 1980). In a later study it was reported that the acetylation of lysines 21 and 75 located in the central helix significantly reduces the affinity of CaM for MLCK and the acetylation of lysines 13, 30, 77, 94, or 148 had only a minor effect (Jackson et al., 1987). In general, not much has been done in studying the role of acetylation of CaM and the published data on the subject are quite poor.

3.2.4.2 Methylation

One of the noteworthy modification on CaM is the trimethylation of Lys115 a process driven by S-adenosyl-L-methionine: calmodulin-lysine N-methyltransferase (CaM KMT) (Roberts et al., 1986, Rowe et al., 1986). The dimethylation of Lys13 in *Paramecium* has been described as well (Schaefer et al., 1987). The role of methylated CaM is still unclear, but it seems that the methylated form of the protein has differential effects on the activation of different CaM targets compared to the non-methylated protein. As for example, it has been reported that the replacement of Lys115 with alanine impairs its ability to activate plant NAD kinase and that the N-methylation of CaM prevents its degradation by ubiquitin-ATP-dependent proteolysis (Gregori et al., 1985). It was also reported that methylated and non-methylated CaM both can activate in the same extent PDE1 (Rowe et al., 1986). Moreover, non-methylated CaM was found in different mammalian tissues (Rowe et al., 1986), and

CaM KMT has been found to be subjected to different developmental regulation in plants (Oh et al., 1992). It has been also reported of a transgenic tobacco plant expressing the CaM mutant K117R, and this mutant plant had decreased growth, pollen viability and seed production (Roberts et al., 1992). In support to that study, recently it was demonstrated that the expression of CaM KMT in *Arabidopsis thaliana* was found to be at its highest levels during seed development, especially in tissues with active auxin signaling (Magnani et al., 2010). Moreover, this group reported that CaM KMT has a role in ABA signaling and auxin response to cold, heat and salt stress, and in return the enzyme is regulated by auxin and abiotic stresses. The authors using a microarray chip containing *Arabidopsis* proteins indentified that the methylated and non-methylated CaM have different binding partners, and that there are methylation-sensitive and methylation-insensitive partners of CaM (Magnani et al., 2010). It seems that N-methylation of CaM is involved in growth, body and seed development, and stress related functions in plants and gives rise to the possibility that it has similar role in animal cells. However, a Lys115 mutant CaM does not have a differential effect compared to wild type CaM on cell proliferation and viability (Panina et al., 2012)

CaM can also undergo carboxyl methylation (Gagnon et al., 1981), the protein itself is very good substrate for protein-carboxyl methyltransferase, as it has many acidic and basic residues and a low isoelectric point (Means and Dedman, 1980). CaM was also found to be carboxy methylated in living cells (Gagnon et al., 1981). The role of this modification is not clear, but it has been reported that carboxy methylated CaM has reduced ability to activate PDE1 *in vitro* and that this modification does not impair the Ca^{2+} binding, but most likely it affects the conformation of the Ca^{2+}/CaM complex (Weiss et al., 1980).

3.2.4.3 Phosphorylation

Reversible protein phosphorylation of threonine, serine, tyrosine or histidine residues is one of the most well studied posttranslational modifications and it is one of the most important in functional terms. Phosphorylation plays crucial roles in regulation of many cellular processes such as cell growth, cell cycle, apoptosis, cell metabolism, and signal transduction among many others (Fischer, 2013).

It has been demonstrated that CaM is phosphorylated both *in vitro* and *in vivo* (Plancke and Lazarides, 1983, Nakajo et al., 1986, Salas et al., 2005, Benaim and Villalobo, 2002). There are four serine and twelve threonine residues in CaM that could be subjected to phosphorylation by protein-Ser/Thr kinases and only two tyrosine residues that could be targeted for phosphorylation by different protein-tyrosine

kinases. Figure 2 shows the 3D structure of CaM from *Homo sapiens* where different amino acids known to be phosphorylated are highlighted.

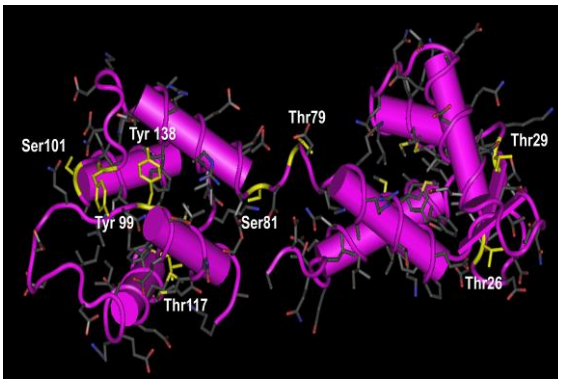

Figure 2: Serine, threonine and tyrosine residues in CaM phosphorylated by different kinases. The figure was prepared using the Cn3D 4.3.1 program and the amino acids phosphorylated by protein-serine/threonine kinases and protein-tyrosine kinases are highlighted in yellow. The CaM structure (pdb code: 1CFD_A) is presented as wire and the side-chains of the amino acids are shown with sticks.

Phosphorylation of CaM by protein Ser/Thr kinases

Four of the threonine residues have been reported to be subject to phosphorylation: Thr26, Thr29, Thr79 and Thr117 (Davis et al., 1996). The first two are phosphorylated by MLCK, the kinase phosphorylates mainly Thr29, while much lower phosphorylation of Thr26 was also detected. Interestingly, the MLCK was able to phosphorylate CaM in a Ca^{2+}-independent manner even when its canonical CaM-BD was mutated (Davis et al., 1996). Thr79 and Thr117 on the other hand are phosphorylated by casein kinase-II (CK-II), and this kinase is also responsible for the phosphorylation of Ser81 and Ser101 (Nakajo et al., 1986, Meggio et al., 1987, Nakajo et al., 1988, Quadroni et al., 1994). Thr79 and Ser81 are located in the central alpha helix of CaM, region with great importance for the binding of CaM-BPs (Figures 1B and 1C, Figure 2). The phosphorylation of Ser101, which is located in the III Ca^{2+}-binding pocket, may not only affect the binding of target proteins but also the binding of Ca^{2+}. Therefore, the effect of CK-II phosphorylation was investigated and two controversial finding were published (Aiuchi et al., 1991, Quadroni et al., 1998). The first one reported that CaM phosphorylated by CK-II has higher affinity for Ca^{2+} compared to non-phosphorylated CaM (Aiuchi et al., 1991). In reverse, the other group using flow dialysis reported that CK-II-phosphorylated CaM has lower affinity for Ca^{2+} (Quadroni et al., 1998). In fact Ca^{2+} at low concentrations stimulates the phosphorylation of CaM by CK-II and has inhibitory effect at higher concentrations (Meggio et al., 1987, Sacks and McDonald, 1992).

Phosphorylation of CaM by receptor and non-receptor protein tyrosine kinases

Unlike Ser/Thr phosphorylation of CaM, where only two kinases are so far known to be involved: CK-II and MLCK, Tyr99 and Tyr138 are targeted by at least eight

different protein-tyrosine kinases (Figure 3). There are only two protein-tyrosine receptor kinases known so far, that are able to phosphorylate CaM: the InsR and the EGFR, and about six non-receptor tyrosine kinases, four of the Src family plus Syk and Jak2 kinases, that also are able to phosphorylate CaM.

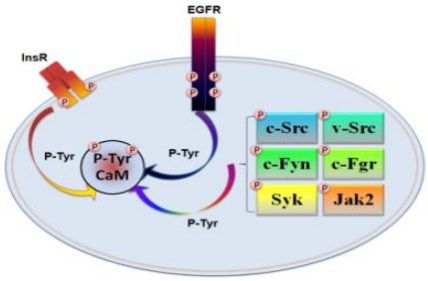

Figure 3: Receptor and non-receptor protein tyrosine kinases known to phosphorylate CaM. The receptor protein-tyrosine kinases EGFR and InsR are shown in their active conformation as well as the non-receptor tyrosine kinases of the Src family (c-Src, v-Src, Fyn and Fgr) plus Syk and Jak2.

Phosphorylation of CaM by InsR

In 1985 the group of McDonald for the first time reported that the InsR from rat adipocyte membranes is a CaM-BP, suggesting that Ca^{2+}/CaM has an important role in insulin-induced events (Graves et al., 1985). One year later the same group reported that CaM is not only binding to the InsR, but also enhances the receptor activity and in return the kinase phosphorylates CaM (Graves et al., 1986, Wong et al., 1988, Sacks and McDonald, 1988, Laurino et al., 1988, Sacks et al., 1989a). Shortly after this discovery a few additional papers were published on the subject, demonstrating that CaM is phosphorylated by the InsR in different tissues such as adipose tissues, skeletal muscle, liver and placenta (Graves et al., 1986, Wong et al., 1988, Sacks and McDonald, 1992, Sacks et al., 1989a). The *in vitro* phosphorylation of CaM by the InsR requires different polycations or basic proteins, such as poly-L-lysine or histones, as co-factors (Sacks and McDonald, 1988) and it has been suggested that in living cells this function could be exerted by the C-terminal of K-Ras. Indeed, a stimulatory effect on CaM phosphorylation by the InsR was demonstrated in an *in vitro* assay using a highly basic segment from K-Ras where the *in vitro* phosphorylation of CaM by InsR was also demonstrated (Fujita-Yamaguchi et al., 1989, Sacks et al., 1989b). Another interesting points on the subject are that low concentrations of Ca^{2+} have a highly stimulatory effect on the phosphorylation of CaM, while higher concentrations inhibits this process (Graves et al., 1986, Sacks and McDonald, 1992, Sacks et al., 1989a, Sacks, 1994), and that the InsR increased its potential to phosphorylate CaM that was previously phosphorylated by CK-II (Laurino et al., 1988). InsR is able to phosphorylate both Tyr99 and Tyr138, nevertheless, there is contradictory information published as some reports suggest that only Tyr99 was phosphorylated (Laurino et al., 1988, Saville and Houslay, 1993, 1994), while others demonstrate that both tyrosines are targeted by the InsR (Wong et al., 1988, Williams et al., 1994).

Phosphorylation of CaM by EGFR

In 1986 the group of Wharton detected phospho-Ser-CaM in EGF-stimulated A431 cells (Lin et al., 1988). However, this was an indirect effect mediated by an unknown Ser/Thr kinase. The direct tyrosine-phosphorylation of CaM by the EGFR was discovered and extensively studied by the group of Villalobo (San José et al., 1992, Benguría et al., 1994, 1995, De Frutos et al., 1997, Palomo-Jiménez et al., 1999, Benaim et al., 1998). It was reported that EGFR purified from rat liver by CaM-affinity chromatography was able to phosphorylate CaM at tyrosine (Benguría et al., 1994). The addition of poly-L-lysine or histones for the *in vitro* phosphorylation of CaM by EGFR, is an important requirement, as in the case with the InsR (Benguría et al., 1994). Interestingly, 1 µM of free Ca^{2+} was sufficient to profoundly inhibit CaM phosphorylation by EGFR (Benguría et al., 1994, De Frutos et al., 1997). Speaking of Ca^{2+} concentration and EGFR-CaM interaction it is of note that one of the consequences of the EGF-mediated activation of the receptor is the increase of cytosolic Ca^{2+} concentration and the extent of this increase maybe equivalent to a gradual decrease in the EGF-dependent phosphorylation of CaM (Villalobo et al., 2000). EGFR predominantly phosphorylates Tyr99, but it is also capable of phosphorylating Tyr138 (Palomo-Jiménez et al., 1999).

Phosphorylation of CaM by non-receptor tyrosine kinases

Several members of the Src family (c-Src, v-Src, Fyn and Fgr) have been shown to phosphorylate CaM as well as other tyrosine kinases such as Jak2 and Syk. It has been reported that CaM is phosphorylated in Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts but not in normal chicken embryo fibroblasts, and purified v-Src has been shown to phosphorylate CaM *in vitro* and that this phosphorylation was inhibited by Ca^{2+} (Ki = 30 µM) (Fukami et al., 1986). An interesting observation is that v-Src phosphorylated CaM had a slower electrophoretic mobility compared to non-phosphorylated CaM (Fukami et al., 1986). We have successfully phosphorylated CaM using recombinant c-Src and the presence of poly-L-lysine or histones strongly enhances the process (Salas et al., 2005, Stateva et al., 2015). However, the subsequent mutagenesis of Tyr99 and Tyr138 to Phe, Glu or Asp showed that when Tyr99 was mutated the efficiency of phosphorylation of Tyr138 was far greater as compared to the phosphorylation of Tyr99 when Tyr138 was mutated. This suggests that Tyr138 is the one preferred by the kinase (Salas et al., 2005, Stateva et al., 2015). Other non-receptor tyrosine kinases from the Src family capable of phosphorylate CaM are c-Fyn and c-Fgr, and both phosphorylate Tyr99 and Tyr138 (Corti et al., 1999).

The other two non-receptor tyrosine kinases known to phosphorylate CaM are Syk and Jak2. Syk kinase from spleen is able to tyrosine phosphorylates CaM at very low concentration of polycations (Meggio et al., 1987). As it is true for the Syk kinase, the information available for Jak2 CaM phosphorylation is scarce and there is only one report demonstrating that Jak2 is in fact capable of phosphorylate CaM (Mukhin et al., 2001).

3.3 CaM-dependent systems

As previously mentioned CaM is a ubiquitously expressed protein that has been found in all eukaryotic cells. Its subcellular localization may vary and it can be found in the cytoplasm, nucleus, attached to the plasma membrane or within organelles, regulating more than 300 different proteins with enzymatic or non-enzymatic functions (Crivici and Ikura, 1995, Shifman et al., 2006, Berchtold and Villalobo, 2014).

3.3.1 Cyclic nucleotide phosphodiesterase 1

PDEs form a large group of enzymes that specifically hydrolyze the second messengers cAMP and cGMP into the non-cyclic nucleotides 5'-AMP and 5'-GMP, respectively (Weishaar, 1986, Halpin, 2008, Keravis and Lugnier, 2012). Immediately after the discovery of cAMP the cyclic nucleotide PDE activity was described (Butcher and Sutherland, 1962). During the next two decades, research into the biochemical characterization and functional role of PDEs has grown exponentially and in this period around 1300 research articles have been published. More and more different PDE were found in different tissues and cell extracts. Interestingly the PDE activity as well as the distribution of PDE differed among different tissues and this led to a huge confusion in the nomenclature. Finally, the different PDEs were classified in 11 families based on the primary amino acid sequence as well as their kinetic and regulatory properties (Omori and Kotera, 2007). All of these 11 families of PDEs have a N-terminal regulatory domain (R-domain) and a C-terminal catalytic domain (C-domain). The N-terminal region of the PDEs differs the most among PDEs in size and structure, and contains sequences that are target for various regulators, and it also contain the binding site for cGMP and CaM, phosphorylation sites, and it is involved in membrane association and dimerization events (Lugnier, 2006).

CaM regulation of PDE1

The existence of Ca^{2+}-stimulated phosphodiesterase activity and that this is mediated by a heat stable protein activator was first demonstrated in the early ’70 of the last century (Cheung, 1970, Kakiuchi and Yamazaki, 1970). Since their discovery Ca^{2+}/CaM-dependent PDEs have been isolated from many mammalian tissues and the richest source of CaM-dependent isoenzyme (PDE1) is the bovine brain, but CaM-dependent PDEs have been isolated also from other tissues such as lung and heart (Sharma and Wang, 1986, Sharma and Kalra, 1994). CaM-dependent PDEs are named depending on their tissue distribution: brain (60 and 63 kDa isoforms), lung and heart, and these isoenzymes have different affinities for cGMP, cAMP and Ca^{2+}/CaM but what is common for all of them is that they have higher affinity for cGMP than for cAMP (Sharma and Kalra, 1994). Nevertheless, the K_m of the different isoenzymes of CaM-dependent PDEs for cAMP and cGMP may vary in a big range, for instance the K_m for cAMP of the enzyme in bovine brain has been shown in deferent studies to be in the range from 10 to 200 µM (Sharma and Kalra, 1994, Morrill et al., 1979, Klee et al., 1979, Tucker et al., 1981, Shenolikar et al., 1985).

The crucial role of Ca^{2+}/CaM in the activation of certain PDE isoenzymes is obvious, as Ca^{2+}/CaM is absolutely necessary for the maximal activation of these enzymes (Kakiuchi and Yamazaki, 1970, Sharma and Kalra, 1994), but this is only at first sight, since Ca^{2+}/CaM has much more to do in regulating this system. The activation of cells upon various stimulus leads to the activation of adenylate cyclase (AC), an enzyme able to convert ATP into cAMP, thus increasing the cellular concentration of cAMP. At the same time the cytosolic concentration of Ca^{2+} also rises and the increased levels of the Ca^{2+}/CaM complex and cAMP concentration activates the PDEs that starts to hydrolase cAMP to AMP. At that point the high levels of cAMP activate protein kinase A (PKA), which in turn phosphorylates PDE inactivating this enzyme. In fact, the inactivation of PDE by phosphorylation is not only due to PKA, the increase of the Ca^{2+} concentration leads to the activation of CaMKKs, which are also able to phosphorylate and inactivate the PDE. During these events the Ca^{2+} continues rising and finally achieves its pick and Ca^{2+}/CaM is now able to activate calcineurin (CaN), which is a ubiquitous phosphatase, that is able to dephosphorylate and activate PDE (Sharma et al., 2006). Taken all together it makes Ca^{2+} and the Ca^{2+}/CaM complex essential regulators of this processes, providing different mechanism for achieving a steady-state equilibrium between activation and inhibition of the main players in this signaling cascade.

Role of phospho-CaM on the regulation of PDE1

It has been reported that CaM phosphorylated by CK-II has lower affinity for bovine brain PDE1 compared to non-phosphorylated CaM, while the affinity of the enzyme for Ca^{2+} and the V_{max} remain the same (Sacks et al., 1992, Quadroni et al., 1998,). This observation was further proved by testing dansylated phospho-Ser/Thr-CaM with a peptide corresponding to the CaM-binding site of the same PDE1, as the phosphorylated peptide had lower affinity for PDE1 compared to the non-phosphorylated one (Leclerc et al., 1999).

The information published about the effect of phospho-Tyr-CaM is not that homogeneous, as some authors detected lower affinity of phospho-Tyr99/Tyr138-CaM (phosphorylated by the InsR) for PDE1 from bovine brain compared to the non-phosphorylated one (Williams et al., 1994), while purified phospho-Tyr99-CaM presented higher affinity (Corti et al., 1999) and in both cases the V_{max} was similar to the one presented by non-phosphorylated CaM. On the other hand, CaM phosphorylated by the EGFR was incapable of activating PDE1 from bovine heart (Palomo-Jiménez et al., 1999), while InsR-phosphorylated CaM has no effect when tested toward PDE1 isolated from rat hepatocytes (Saville and Houslay, 1994). The role of phospho-Tyr-CaM on PDE1 seems to fluctuate depending on the purity of the phospho-Tyr-CaM preparation, the Tyr residues subject to phosphorylation, as well as the isoform of the enzyme.

3.3.2 Nitric oxide synthase

Nitric oxide (·NO) is a very important free radical second messenger molecule controlling process such as the immune response, blood pressure and neuronal activity among others (Kerwin et al., 1995, Bogdan, 2015, Das and Kumar, 1995, Luo and Zhu, 2011). ·NO is produced by the enzyme nitric oxide synthase (NOS) as product of the conversion of L-arginine into L-citrulline. For each L-citrulline and ·NO produced, 2 molecular oxygens and 1.5 NADPH are consumed (Kerwin et al., 1995).

NOS isoforms

There are three isoforms of mammalian NOS: endothelial (eNOS) expressed mainly in endothelial cells, neuronal (nNOS) expressed mainly in neuron cells and inducible (iNOS) expressed in macrophages, but can be induced in many other cell types (Griffith and Stuehr, 1995). The ·NO produced by eNOS controls vascular dilation and blood flow (Das and Kumar, 1995), nNOS-produced ·NO is responsible for

neurotransmission (Luo and Zhu, 2011) and the one produced by iNOS is used in the immune system as a defense mechanism (Bogdan, 2015). eNOS and nNOS are constitutively expressed and are classified as constitutive NOS (cNOS). Their enzymatic activity depends on increasing Ca^{2+} concentration in the cell, due to the Ca^{2+}-dependent binding of CaM to these isoforms (Govers and Rabelink, 2001). iNOS, on the other hand, is expressed only when required and as soon as it is transcribed, it can bind to CaM in the absence or presence of Ca^{2+}, and CaM is a constitutive subunit of the enzyme (Cho et al., 1992). The overall structure of the three isoforms is similar, with an oxygenase domain and a reductase domain separated by a CaM-BD. The oxygenase domain contains binding sites for tetrahydroxybiopterin (TH4), the catalytic heme and the active site, and the reductase domain accommodates the binding sites for NADPH, FAD and FMN. The NOS enzymes are found to be homo-dimeric proteins (Alderton et al., 2001).

CaM regulation of NOS

CaM is essential for the activation of the NOS isoforms. The binding affinity of CaM for each of the isoforms differs, with the highest binding affinity for iNOS, followed by nNOS and then eNOS (Aoyagi et al., 2003, Ghosh and Salerno, 2003). CaM binds to NOS at a 1-5-8-14 CaM-binding motif with the binding region consisting of residues 731-752 of nNOS, 491-512 of eNOS, and 501-531 of iNOS. It is believed that iNOS binds CaM in a Ca^{2+}-independent manner, however no IQ-motif within the protein has been identified so far. It is believed that CaM binds to iNOS in both Ca^{2+}-dependent and Ca^{2+}-independent manners (Aoyagi et al., 2003). A conformational change that is associated with CaM binding to NOS is required for electron transfer within these enzymes (Govers and Rabelink, 2001). The conformational change that CaM induces in the reductase domain of NOS allows for the FMN domain to interact with FAD to accept electrons and pass the electrons to the heme during catalysis. Exactly how CaM is able to initiate this process is still not fully understood. Clearly, these conformational changes caused by CaM are important for stimulating efficient electron transfer within the NOS enzymes (Daff et al., 2001).

Role of phospho-CaM on the regulation of NOS enzymes

Carafoli's group have demonstrated that phospho-(Tyr)-CaM and phospho-(Ser/Thr)-CaM both have stimulatory effects on the activation of NOS isolated from brain compared to the non-phosphorylated protein (Quadroni et al., 1994, Corti et al., 1999). Titration assay of dansyled CaM and dansyled phospho-(Ser/Thr)-CaM with a

peptide corresponding to the CaM-BD of NOS exhibited lower affinity for the phosphorylated than for the non-phosphorylated form (Leclerc et al., 1999). This observation was further confirmed and it was reported that CK-II-phosphorylated CaM has lower ability to activate eNOS and has two fold reduced V_{max} compared to non-phosphorylated CaM, while the affinity of the enzyme for CaM and Ca^{2+} was not changed (Greif et al., 2004).

In terms of phospho-(Tyr)-CaM and its effect on NOS activation not much has been done during the last few years. What we know is that in a displacement assay of a dansylated peptide corresponding to the CaM-BD of the Ca^{2+}-ATPase, which is bound to non-phosphorylated CaM or phospho-(Tyr99)-CaM by a peptide corresponding to the CaM-BD of NOS showed that the affinity of phospho-(Tyr99)-CaM for the CaM-BD of NOS was higher compared to non-phosphorylated CaM (Corti et al., 1999).

3.3.3 Tyrosine-protein kinase c-Src

c-Src kinase belongs to the Src family of non-receptor tyrosine kinases. Src family kinases are regulating processes such as cell differentiation, proliferation, motility and cell survival, stress response among other processes. It comprises Blk, Fgr, Fyn, Lck, Lyn, Hck, Yes, and c-Src itself. Fgr, Blk and Hck are found mainly within blood cells; Lyn and Lck in blood cells and brain, while Yes, Fyn and c-Src are ubiquitously expressed (Parsons and Parsons, 2004, Yeatman, 2004). The 3D structure of c-Src is well studied. c-Src is a 60 kDa modular protein formed by a N-terminal region containing a myristoyl-binding sequences, a SH4 domain, an Unique domain (UD), SH3 and SH2 domains followed by the protein-tyrosine kinase domain (SH1) and a short C-terminal tail (Xu et al., 1997). Src family members are attached to the cellular membrane through myristoylation in its N-terminal (Patwardhan and Resh, 2010). c-Src and oncoprotein v-Src kinases play prominent roles in tumor progression and metastasis (Parsons and Parsons, 2004, Wheeler et al., 2009), thus they have been targeted by different inhibitors and strategies in thyroid, pancreas, and breast cancers (Wheeler et al., 2009).

The non-receptor tyrosine kinase Src is subjected to complex regulatory mechanisms mediated by phosphorylation events that control its activation status (Okada and Nakagawa, 1989, Cooper and Howell, 1993, Parsons and Parsons, 2004, Irtegun et al., 2013). When inactive, c-Src is phosphorylated at Tyr530 (Tyr527 in chicken) by the tyrosine kinases Csk and Chk (Chong et al., 2005). Tyr530 is a highly conserved residue among all Src kinase family members and it is located at the C-

terminal tail of the protein. When Tyr530 is phosphorylated the C-tail interacts with the SH2 domain and the kinase is in its “closed” and inactive conformation (Ayrapetov et al., 2006, Roskoski, 2005). Dephosphorylation of Tyr530 by tyrosine phosphatases induce conformational changes and the subsequent auto-phosphorylation of Tyr419 opens up the kinase and stabilizes the catalytic domain in an active state (Roskoski, 2005).

CaM regulation of c-Src

Hayashi *et al.* demonstrated that a myristoylated peptide corresponding to the N-terminal of v-Src interacts with the Ca^{2+}/CaM complex, while the non-myristoylated peptide was not able to do so (Hayashi et al., 2004). It has been also proposed that upon Ca^{2+} entry in stimulated cells, CaM residing in lipid rafts interacts in a Ca^{2+}-dependent manner with c-Src, and in turn c-Src phosphorylates and inhibits PP2A, preventing in this manner its inhibitory action on Akt and henceforth promoting melanoma tumor growth (Fedida-Metula et al., 2012). The activation of c-Src by Ca^{2+}/CaM was also demonstrated in transfected neuroblastoma cells overexpressing α-synuclein, a cytotoxic protein abundant in Lewy bodies in Parkinson’s disease, by a mechanism also implicating PP2A (Yang et al., 2013). The direct interaction of CaM with a recombinant GST-Src fusion protein was demonstrated to occur through both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, although mutation of the proposed CaM-binding site located at the SH2 domain of c-Src only partially prevented CaM binding (Yuan et al., 2011). The most recent evidence of CaM binding to Src surprisingly involves the Unique domain of the kinase. Pérez *et al.* using NMR and peptides corresponding to the Unique and SH3 domains of Src have demonstrated that Ca^{2+}/CaM is able to bind to the Unique domain and thus modulating the binding of the kinase to the plasma membrane (Pérez et al., 2013). Additionally, Src was shown to co-immunoprecipitate with CaM but not with tyrosine-phosphorylated CaM in keratinocytes (Wu et al., 2015).

Nevertheless, these reports did not fully clarify the actual mechanism by which CaM interacts and activates c-Src, or if this process always occurs in the cell in a Ca^{2+}-dependent manner. This highlights the need for additional work to determine whether CaM controls the tyrosine kinase activity of c-Src in both Ca^{2+}-dependent and/or Ca^{2+}-independent manners. There is no information about the role of the phospho-CaM on c-Src functionality so far.

3.3.4 Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein, and it belongs to the ErbB receptor tyrosine kinase family formed by ErbB1/EGFR/Her1, ErbB2/Neu/Her2, ErbB3/Her3 and ErbB4/Her4. Its structure consists of an extracellular region which is responsible for ligand-binding, a single transmembrane segment and a cytosolic region that contains a juxtamembrane domain (JD), a tyrosine kinase domain (TK) and a C-terminal tail (Figure 4) (Schlessinger, 2014, Olayioye et al., 2000, Yarden, 2001). A general accepted mechanism of EGFR activation is that upon binding of ligands, including EGF (epidermal growth factor), TGF-α (transforming growth factor-α), HB-EGF (heparin-binding EGF-like growth factor), BTC (betacellulin), AREG (amphiregulin) and EPR (epiregulin) causes structural changes of the domains, and induces dimerization of two receptor monomers (Olayioye et al., 2000, Yarden and Sliwkowski, 2001, Ferguson et al., 2003). EGFR can form homo-dimers or hetero-dimers with other ErbB family members (Olayioye et al., 2000, Gullick, 2001). The dimerization leads to activation of the intrinsic tyrosine kinase domain followed by auto-(trans)-phosphorylation at multiple Tyr residues located in the C-terminal tail: Y992, Y1045, Y1068, Y1086, Y1148 and Y1173 (Linggi and Carpenter, 2006). These auto-phosphorylation sites then are docking sites for different cytosolic proteins containing SH2 (Src homology 2) and PTB (phosphotyrosine binding) that are adaptors and signaling proteins forming transduction complexes to activate signaling pathways such as the RAF-RAS-MEK-ERK, the PI_3K-Akt, the PLCγ-PKC, and the STATs pathways (Figure 4). The activation of these signaling pathways results in cell cycle progression, cell proliferation, migration, adhesion, survival, and differentiation (Schlessinger, 2014, Yarden, 2001, Wagner et al., 2013).

Ligand-induced EGFR internalization and degradation have been extensively studied (Wiley, et al., 1991, Kirisits et al., 2007, Zwang and Yarden, 2009). Following activation EGFR undergoes internalization through endocytosis where the receptor-ligand complex is sorted either for degradation or recycling back to the plasma membrane, or for nuclear translocation (Kirisits et al., 2007, Henriksen et al., 2013). After ligand binding, EGFR associates to coat adaptors such as adaptor protein 2 and Eps15 followed by endocytosis of the receptor through clathrin-coated pits, caveolae-mediated endocytosis, or through macropinocytosis (Zwang and Yarden, 2009). EGFR is frequently overexpressed, miss-regulated or mutated in many solid tumors contributing to cancerogenesis, thus the receptor has became an important target for cancer therapy (Yarden, 2001, Schlessinger, 2014, Lemmon et al., 2014, Dancey, 2004).

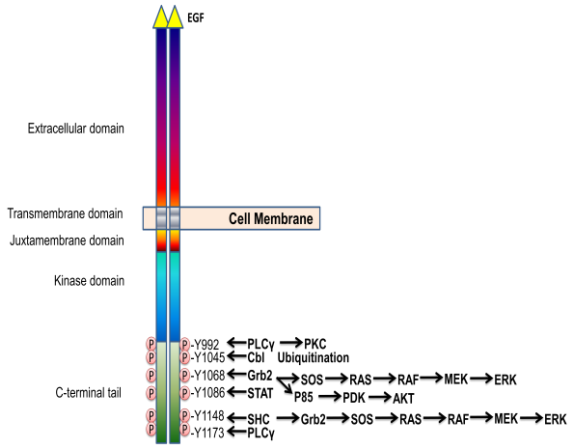

Figure 4: EGFR signaling pathways. Schematic representation of the functional domains of EGFR, the tyrosine phosphorylation residues induced by ligand (yellow triangle) stimulation and some downstream signaling cascades.

CaM regulation of the EGFR

The direct interaction of CaM with the EGFR was first reported in several studies done by our group (San José et al., 1992, Martín-Nieto and Villalobo, 1998, Li et al., 2004, 2012, Sengupta et al., 2007). The first evidence of direct binding between these two proteins was the identification of solubilized EGFR from rat liver bound to CaM-Sepharose in the presence of Ca^{2+} (San José et al., 1992). Later on the same was replicated using human EGFR, overexpressed in stably transfected murine fibroblasts (Martín-Nieto and Villalobo, 1998). In recent years the JM domain has been shown to play an important role in the regulation of the receptor and the specificity of its signaling (Hubbard, 2009, Red Brewer et al., 2009, Jura et al., 2009). In the JM region was identified a basic amphiphilic site comprising amino acids ^{645}RRRHIVRKRTLRRLLQ^{660} with CaM-binding properties (Martín-Nieto and Villalobo, 1998). Experiments using the CaM-BD of EGFR tagged with GST, determined that the interaction between CaM and the EGFR JM occurred in a Ca^{2+}- dependent manner and the formation of the complex was abolished in the presence of the Ca^{2+}-chelating agent EGTA (Martín-Nieto and Villalobo, 1998). In a more recent paper published by our group the role of CaM binding on EGFR functionality was further supported in living cells (Li et al., 2012). We used a conditional CaM-KO cell line in which all alleles of the genes encoding endogenous CaM were deleted and a construct encoding CaM from *Rattus norvegicus* was inserted. The latter one bearing a Tet-off cassette allowing the suppression of the expression of rat CaM in those cells by the addition of tetracycline (Panina et al., 2012). This cell line was stably transfected with the human EGFR and it was demonstrated that after the suppression of the expression of CaM the EGF-dependent activation of the receptor was significantly reduced highlighting the importance of CaM binding for EGFR functionality (Li et al., 2012).

Several models for how CaM mediates the regulation of the EGFR have been proposed (McLaughlin et al., 2005, Sánchez-González et al., 2010). McLaughlin et al. suggested that the basic amino acids that compose the cytosolic JM and the tyrosine kinase domain of the receptor is attached to the acidic lipids in the inner leaflet of the plasma membrane of the cell through electrostatic interaction and serves as an autoinhibitory mechanism of the receptor in the absence of ligand (McLaughlin et al., 2005). Ligand binding to the receptor leads to a rapid increase in the amount of the cytosolic Ca^{2+}, thus inducing the formation of the Ca^{2+}/CaM complex, which binds to the CaM-BD of the JM domain of the EGFR and detach it from the membrane. The detachment of the JM domain now allows the receptor to form an active homo-dimer (Figure 5) (McLaughlin et al., 2005, Sato et al., 2006).

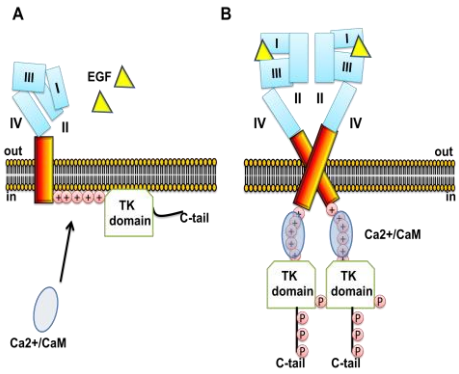

Figure 5: Model for the activation of the EGFR by the Ca^{2+}/CaM complex. (A) EGFR in an inactive state. The cytosolic JM is bound to the plasma membrane followed by the tyrosine kinase domain (TK) domain, which is also close to the negatively charged membrane. (B) EGF activated EGFR: Ca^{2+}/CaM binds to the CaM-BD in the JM and pull it out from the negatively charged bilayer. Adapted from (McLaughlin et al., 2005, Sato et al., 2006).

However, more recent NMR studies have suggested that the transmembrane domain dimerization is most likely enough to trigger the dissociation of the JM from the membrane (Matsushita et al., 2013). However, all of these models are based on the possibility that the membrane integrity is necessary to hold the receptor in an autoinhibited state in the absence of its ligand (Lemmon et al., 2014).

Phospho-CaM regulation of the EGFR

The effect of phospho-(Y)-CaM and phospho-(Ser/Thr)-CaM on the EGFR functionality is still not clear. To our knowledge nothing has been done with the phospho-(Ser/Thr)-CaM, while our group have shown that phospho-(Tyr)-CaM in both the absence and presence of Ca^{2+} have an activating role in the EGF-dependent EGFR activation of the solubilized receptor *in vitro*, while non-phosphorylated CaM had an inhibitory effect in the presence of EGF in this *in vitro* assay system (Stateva et al., 2015, manuscript in preparation).

3.4 *O*-GlcNAcylation

Protein *O*-GlcNAcylation is a posttranslational modification first identified by Hart group in 1984 in lymphocyte proteins (Torres and Hart, 1984). It consists in the reversible *O*-linked attachment of single β-D-*N*-acetylglucosamine moieties to serine/threonine residues in target proteins, but unlike the other types of glycosylation it is not further extended to more complex structures and occurs predominantly in intracellular proteins (Holt and Hart, 1986). It was shown that this modification has a short half-life and it can respond to different stimuli (ligand binding or different type of stress) within minutes (Zachara and Hart, 2004, Kneass and Marchase, 2004). Since then *O*-GlcNAcylation has been indentified in all higher eukaryotes from *Caenorhabditis elegans* to human and found to modify hundreds of different proteins, modifying their enzymatic activity, intracellular location, turnover, and/or exerting other functional roles (Hart, 2014). Altered *O*-GlcNAcylation has been proposed to contribute to the etiology of many illnesses such type-2 diabetes (Lefebvre et al., 2010, Hart et al., 2011, Ma and Hart, 2013, Vaidyanathan and Wells, 2014), cardiovascular ailments (Lefebvre et al., 2010, Marsh et al., 2014), neurodegeneration as in Alzheimer's disease (Gong et al., 2006, Zhu et al., 2014) and cancer (Li and Yi, 2014, Jozwiak et al., 2014, de Queiroz et al., 2014, Ma and Vosseller, 2014). The enzyme that transfers GlcNAc moieties to proteins is *O*-linked β-*N*-acetylglucosamine transferase (OGT) and unlike the rest of the enzymes involved in glycosylation is not restricted to the endoplasmic reticulum or Golgi (Janetzko and Walker, 2014). Three OGT isoforms are present in the cell, a canonical long form localized in the nucleus and cytosol (ncOGT), another one a bit shorter localized in the inner mitochondrial membrane (mOGT), and a third one, the shortest of them (sOGT), also localized in the nucleus and cytosol. These isoforms are basically differentiated by the number of tetratricopeptide (TRP) repeat motifs present in its N-terminal end (Janetzko and Walker, 2014). Although there is no consensus sequence for *O*-GlcNAcylation, OGT may modulate its substrate specificity through its TRP repeats (Iyer and Hart, 2003). It has been also shown that the TRP-rich domain of OGT is able to bind at the C-terminal of the transcriptional co-regulator host cell factor-1 (HCF-1) and subsequently to catalyze its proteolysis (Capotosti et al., 2011, Lazarus et al., 2013). In addition to OGT, an atypical EGF-like-repeat domain specific OGT (EOGT) was found to be also able to *O*-GlcNAcylate the side chain of Ser/Thr at the extracellular segment of different proteins such as Dumpy in *Drosophila* and the Notch receptor in mouse (Sakaidani et al., 2011, Sakaidani et al., 2012, Ogawa et al., 2014). Interestingly, the *O*-GlcNAcylation of the Notch receptor is followed by the addition of galactose, generating an *O*-LacNAc moiety (Sakaidani et al., 2012).

The enzyme that removes the *O*-GlcNAc from the protein backbone is the *O*-linked β-*N*-acetylglucosaminidase (OGA) that unlike the classical hexosaminidases that are resident in the lysosomes it has been found to be distributed in the nucleus and in the cytoplasm (Dong and Hart, 1994, Alonso et al., 2014). OGA shares significant sequence similarities with acetyltransferases, and appears to be a bifunctional enzyme. In fact, it has been shown to have acetytransferase activity *in vitro* (Toleman et al., 2004). It has been demonstrated that overexpression of OGA induces early mitotic exit and disruption of phosphorylation events during mitosis, while inhibiting it induces growth arrest (Slawson et al., 2005).

Protein *O*-GlcNAcylation is mainly intracellular, dynamic and it cycles on Ser/Thr residues in a manner very similar to protein phosphorylation. Interestingly the scientists have found out that there are extensive crosstalks between both PTMs, they either take place at the same site, or at distinct adjacent sites, competing or facilitating each other (Hart et al., 1995, Mishra et al., 2011, Zeidan and Hart, 2010). This crosstalk has been found to be involved in the regulation of multiple pathways implicated in the physiology of the cell, such as: metabolic control, stress response, epigenetic modification, gene transcription, translation of proteins, protein turnover, differentiation, apoptosis and cell cycle control among others (Wang et al., 2008, Zeidan and Hart, 2010, Hart et al., 2011, Lewis and Hanover, 2014). Interestingly, *O*-GlcNAcylation of some proteins can also affect its Tyr phosphorylation, as it is the case of prohibitin (Ande et al., 2009), a pleiotropic protein regulating many cellular functions including: proliferation, apoptosis, senescence, development, gene transcription, mitochondrial protein folding and also acting as a cell-surface receptor (Mishra et al., 2010, Theiss and Sitaraman, 2011, Whelan et al., 2010).

In recent years there was significant advancement in the field of *O*-GlcNAcylation and the identification of this process is becoming more available (Hart, 2014). Like for example, it was demonstrated using microarrays that a large set of serine/threonine- and tyrosine-kinases were good substrates for OGT and 42 of a total of 152 candidate kinases were found to be *O*-GlcNAcylated *in vitro* (Dias et al., 2012). Advances in the methodology of protein labeling and recent improvements in Mass Spectrometry (MS) techniques allowed scientists to detect and extensively investigate *O*-GlcNAcylation in many different proteins (Hahne and Kuster, 2011). Sprung *et al.* using high-throughput proteomic analysis and labeling proteins of *Drosophila melanogaster* with the GlcNAc analogue azido-GlcNAc indentified many proteins that are potentially *O*-GlcNAcylated, and interestingly the EGFR type III was among them, suggesting that it was susceptible to undergoes *O*-GlcNAcylation (Sprung et al., 2005). Thereafter, in an *in silico* study it

was proposed that the human EGFR could be subjected to *O*-GlcNAcylation at Thr654 and Ser1046/Ser1047 (Kaleem et al., 2009), phosphorylation sites of protein kinase C (PKC) (Hunter et al., 1984) and CaM-dependent protein kinase II (Countaway et al., 1992), respectively (Figure 6).

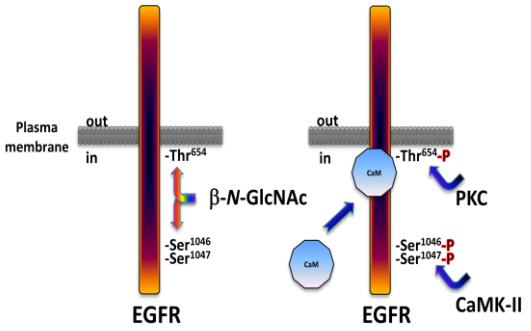

Figure 6: Potential *O*-GlcNAcylation sites in the EGFR based on *in silico* studies. Potential cross-talk between EGFR *O*-GlcNAcylation, and phosphorylation of Thr654 by PKC and Ser1046/Ser1047 by CaMK-II, and occlusion of Thr654 by CaM binding to the receptor at its cytosolic juxtamembrane region (Kaleem et al., 2009).

In fact, both residues Thr654 and Ser1046/1047 are very important control elements, regulating the functionality of the receptor. The generally accepted theory about the role of the phosphorylation of Thr654 by PKC is that this modification leads to the so-called desensitization or "shut-down" of the receptor followed by compartmentalization, internalization and recycling of the EGFR (Countaway et al., 1992, Llado et al., 2004). A more recent study, however, showed that c-Cbl-induced ubiquitination of EGFR and subsequent transfer of the receptor from the early to the late endosomes was inhibited by PKC-mediated phosphorylation and that receptor degradation and down-regulation were significantly reduced (Bao et al., 2000). Of note is to mention that CaM is not able to bind to the CaM-BD of the EGFR when the receptor is phosphorylated at Thr654 (Martin-Nieto and Villalobo, 1998), which let Kaleem *et al*. to speculated that the *O*-GlcNAcylation of Thr654 permit CaM to bind and prevents the PKC-mediated phosphorylation of that residue and thus allowing CaM and c-Cbl (multi-adaptor protein with E3-ubiquitin ligase) to guide the receptor degradation (Kaleem et al., 2009). Interestingly, the other two sites indentified as putative sites for *O*-GlcNAcylation, Ser1046/1047, and possible interplay with phosphorylation on the EGFR, are also under the control of Ca^{2+}/CaM, as they are phosphorylated by CaMK-II and this phosphorylation leads to the desensitization of the receptor (Countaway et al., 1992). Kaleem et al. suggested that the *O*-GlcNAcylation of these two sites could activate the receptor. Moreover, in another study it was demonstrated that the InsR, also a transmembrane protein-tyrosine kinase receptor was subjected to *O*-GlcNAcylation (Yang et al., 2008), highlighting that this modification is not restricted only to nuclear and cytosolic proteins. All taken together comes to show the necessity of studying the possible role of *O*-GlcNAcylation on human EGFR, and to explore the possible crosstalk between OGT, CaM, PKC, and CaMKs.

4. OBJECTIVES

1. To study the possible differential role of phospho-(Y)-CaM and non-phosphorylated CaM on different CaM-dependent systems using phospho-(Y)-mimetic CaM mutants:

    - To generate and characterize phospho-(Y)-mimetic CaM mutants and to study their physicochemical properties.

    - To test the phospho-mimetic properties of these CaM mutants on different CaM-dependent systems: PDE1, eNOS, c-Src and EGFR.

2. To investigate the possible role of apo-CaM and Ca^{2+}/CaM on c-Src functionality *in vitro* and in living cells.

3. To study the potential *O*-GlcNAcylation of the EGFR as it has been suggested that it may occur at sites regulated either directly or indirectly by CaM.

4. To study whether *O*-linked β-*N*-acetylglucosamine transferase (OGT) is a CaM-binding protein.

5. Materials & Methods

5.1 Antibodies

Anti-mouse IgG (Fc specific) horseradish peroxidase (HRP)-conjugated secondary antibody, mouse monoclonal anti-tubulin-α (DM1A) antibody, purified mouse IgG (technical grade) and rabbit polyclonal anti-phospho-Src (Y418) (recognizing human phospho-Y416) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Mouse monoclonal anti-phospho-tyrosine antibody (4G10, isotype IgG2bκ), and rabbit monoclonal anti-EGFR (E235) antibody (recognizing the C-terminal domain of EGFR) were obtained from Millipore (Billerica, MA). Anti-rabbit IgG horseradish peroxidase-linked secondary antibody was purchased from Invitrogen (Eugene, OR). Rabbit monoclonal anti-Src (human) (clone 36D10, isotype IgG), rabbit polyclonal anti-phospho-Src family (Y416) and rabbit monoclonal anti-GAPDH (clone 14C10, isotype IgG) antibodies were obtained from Cell Signaling Company. Mouse monoclonal anti-*O*-GlcNAc monoclonal antibody (CTD110.6) and goat anti-mouse IgM (μ heavy chain) HRP-conjugated secondary antibody were obtained from Thermo Scientific-Pierce (Rockford, IL). The anti-*O*-GlcNAc antibody (RL2) was obtained from Novus Biologicals (Littleton, USA). Anti-OGT antibody (ab50271) was obtained from Abcam (Cambridge, UK).

5.2 Reagents and Materials

Phenyl-Sepharose 6 (fast flow), Sepharore 4B, (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride, FMN, FAD, cAMP, 3',5'-cyclic nucleotide PDE1 (from bovine brain), 5'-nucleotidase (from *Crotalus atrox* venom), recombinant GST-tagged human EGFR (695-end) expressed in baculovirus infected Sf9 cells, eNOS (bovine recombinant baculovirus-expressed in Sf9 cells), histone (type II-A), ATP (sodium salt), 4-amino-5-(methylphenyl)-7-(t-butyl)pyrazolo-(3,4-d)pyrimidine (PP1), benzyl 2-acetamido-2-deoxy–α–D-galactopyranoside (BADGP), O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenyl carbamate (PUGNAc), (3aR,5R,6S,7R,7aR)-2-(ethylamino)-3a,6,7,7a-tetrahydro-5-(hydroxymethyl)-5H-pyrano[3,2-d]thiazole-6,7-diol (Thiamet G), tunicamycin, tetracycline, biotin-labeled WGA and UDP-GlcNAc (sodium salt) were obtained from Sigma-Aldrich Co. (St. Louis, MO). The ultrasensitive colorimetric assay kit for eNOS was purchased from Oxford Biomedical Research (Rochester Hills, MI). The QuickChange® XL site-directed mutagenesis kit was acquired from Agilent Technology (Santa Clara, CA), and the QIAprep plasmid preparation kit from Quiagen Ltd. (Manchester, UK). The Slide-A-Lyzer dialysis cassettes and the Classic Magnetic IP/Co-IP kit were purchased from Thermo Scientific-Pierce (Rockford, IL). Competent *Escherichia coli* BL21(DE3)pLysS was purchased from Stratagene (La Jolla, CA), and BugBuster® Ni-NTA His•Bind®

purification kit and purified 6His-tagged recombinant human c-Src expressed by baculovirus in Sf21 insect cells were obtained from Merck/Millipore (Darmstadt, Germany). The ECL kits were obtained from GE Healthcare-Amersham.The PVDF membranes were obtained from Pall Corporation (Dreieich, Germany). Streptavidin peroxidase-conjugated tetraacetylated N-azidoacetylglucosamine (GlcNAz) and Click-iT®-protein reaction buffer kit were obtained from Invitrogen (Eugene, OR). Peptide-N-glycosidase F (PNGase F) (from *Flavobacterium meningosepticum*), *O*-glycosidase cloned from *Enterococcus faecalis* and expressed in *Escherichia coli* (*E. coli*) and neuraminidase cloned from *Clostridium perfringens* and overexpressed in *E. coli* were obtained from New England BioLabs (Herts, UK). Other chemicals used in this work were of analytical grade. The prokaryotic expression vector encoding full-length human OGT was a kind gift of Prof. Suzanne Walker (Harvard Medical School, Boston, MA). The pETCM vector encoding calmodulin was kindly provided by Prof. Nobuhiro Hayashi from the Institute for Comprehensive Medical Science, Fujita Health University, Japan.

5.3 Generation of calmodulin mutants

PCR-aided site-directed mutagenesis was performed in the pETCM vector containing the coding sequence of *Rattus norvegicus* CaM gene II (Hayashi et al., 1998) as template using the QuickChange® XL Site-Directed Mutagenesis kit and the following set of complementary oligos (Table 2).

Mutation	Primer Sequence (5'- 3')
Y99D	5'-GGCAATGGCGACATCAGTGCAGCA-3; 5'-TGCTGCACTGATGTCGCCATTGCC-3'
Y138D	5'-GGGGATGGTCAGGTAAACGACGAAGAGTTTGTACAAATG-3' 5'-CATTTGTACAAACTCTTCGTCGTCGTTTACCTGACCATCCCC-3'
Y99E	5'-GGCAATGGCGAGATCAGTGCAG-3'; 5'-CTGCACTGATCTCGCCATTGCC-3'
Y138E	5'-GGGGATGGTCAGGTAAACGAGGAAGAGTTTGTACAAATG-3' 5'-CATTTGTACAAACTCTTCCTCGTCGTTTACCTGACCATCCCC-3'

Table 2: Primers used in this study.

Sequential mutagenesis was carried out to obtain the double substitutions Y99D/Y138D and Y99E/Y138E using the Y99D and Y99E mutated vectors, respectively, as template. *E. coli* DH5α was transformed with the pETCM, pETCM(Y99D), pETCM(Y138D), pETCM(Y99D/Y138D), pETCM(Y99E), pETCM(Y138E) or pETCM(Y99E/Y138E) vectors for their replication, and purification by the alkaline lysis method (Birnboim and Doly, 1979) using the QIAprep plasmid preparation kit. The correctness of the mutagenesis procedures was ascertained by sequencing the vectors using an oligo annealing to the T7 promoter (Figure 7).

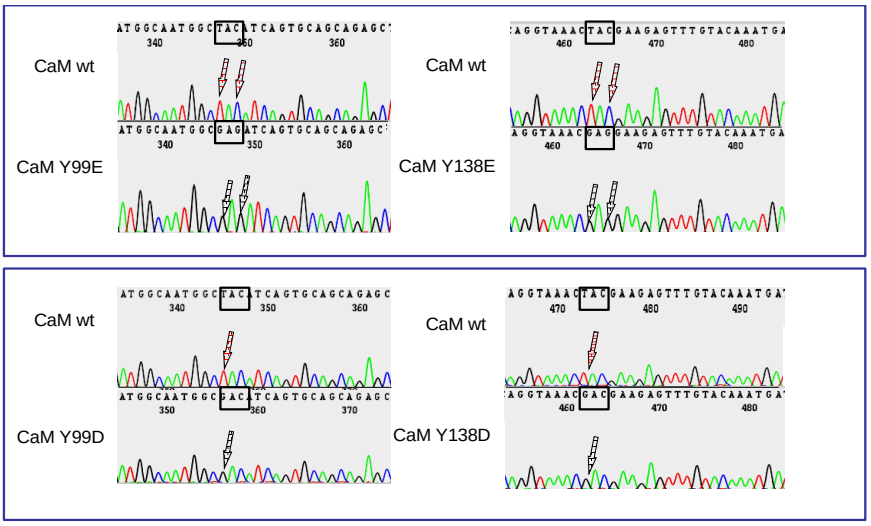

Figure 7: Sequence analysis of CaM(Y99E/D) and CaM(Y138E/D). The substitution of TAC (Tyr) to GAG (Glu) and TAC to GAC (Asp) codons are highlighted and arrows pointing the substitution are also shown. The alignment of the sequences was done with the CodonCode Aligner program.

5.4 Expression and purification of recombinant calmodulin mutants

E. coli BL21(DE3)pLysS was transformed with the vectors indicated above using a thermic-shock protocol. Single colonies grown in solid medium in the presence of 100 μg/ml ampicillin were collected and seeded in 5 ml of Luria´s broth containing the same concentration of ampicillin. Larger cultures (500 ml) were seeded with the pre-culture and grown in the same conditions until they reached an $OD_{600\ nm}$ = 0.7 ± 0.1. The expression of the recombinant proteins was induced with 0.5 mM IPTG during 4 hours at 37 ºC. Control cultures in the absence of IPTG were included. The bacteria were collected by centrifugation at 6,000 x g for 20 min and frozen at -70 °C until used. The bacteria were lysed using the BugBuster® protein extraction reagent kit, centrifuged at 11,000 x g during 10 min at 4 °C and the supernatant collected, heated at 95 °C for 5 min and centrifuged again as above. Heat-resistant CaM present in the new supernatant was purified as previously described (Hayashi et al., 1998). Briefly, phenyl-Sepharose 6 (fast flow) was packed in a column and equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5) and 1 mM $CaCl_2$. The sample was applied to the column and washed with four column volumes of a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ and 100 mM NaCl. CaM was eluted with a buffer containing 50 mM Tris-HCl (pH 7.5) and 2 mM EGTA in fractions of 10 ml. The fractions containing CaM were desalted using Slide-A-Lyzer dialysis cassettes with membranes of 3.5 kDa cut-off against a buffer containing 10 mM Hepes-NaOH (pH 7.5) or 20 mM Tris-HCl (pH 7.5) and kept at a concentration of 1-2 mg/ml at -70 °C. Protein concentration was determined by the BCA method (Stoscheck, 1990) using bovine serum albumin as

standard. The purity of the samples was confirmed by SDS-PAGE (Laemmli, 1970) in a 5-20 % (w/v) polyacrylamide continuous gradient slab gel adding 5 mM EGTA or 1 mM $CaCl_2$ in the loading buffer to attain the characteristic Ca^{2+}-induced electrophoretic mobility shift of CaM (Burgess et al., 1980), and staining the gel with Coomassie Brilliant Blue R-250.

5.5 Absorption spectra

The absorption spectra in the range 240-340 nm of the different species of CaM (120 μM) were measured in 20 mM Tris-HCl (pH 7.5) using a CARY/1E/UV-Visible spectrophotometer (Varian) equipped with Cary winUV software.

5.6 Circular dichroism

Far-UV (200-260 nm) and near-UV (250-320 nm) circular dichroism spectra of the different CaM species were measured at 20 ºC in the presence of either 1 mM EGTA or 1 mM $CaCl_2$ in a buffer containing 20 mM Tris-HCl (pH 7.5) and 0.1 mM KCl using a JASCO J-810 spectropolarimeter (Cremelia, Italy) equipped with a Peltier type temperature control system (Model PTC-423S/L), a bandwidth of 0.2 nm and a response time of 4 s. Far-UV spectra were recorded in 0.1 cm path-length quartz cells at a protein concentration of 0.2 mg/ml, and near-UV spectra in 1 cm path-length cuvettes at a protein concentration of 2 mg/ml as previously described (Sun et al., 2001). The Spectra Manager software (JASCO, v 1.52.01) was used for data collection and analysis. Routinely, the corresponding buffer baseline was subtracted and the corrected data were normalized per mole of amino acid. Thermal denaturation experiments were carried out in the same system by increasing the temperature from 20 to 90 °C at a scanning rate of 0.66 °C/min. Variations in ellipticity at 222 nm were monitored at steps of 0.2 °C.

5.7 Tb^{3+} fluorescence

The emission fluorescence spectra of Tb^{3+} in the range 520-580 nm in the different species of CaM (10 μM) upon excitation of tyrosine (if present) at 280 nm were measured in 10 mM Pipes-HCl (pH 6.5), 100 mM KCl, and increasing concentrations of $TbCl_3$ (0-200 μM) using a Photon Technology International Inc. spectrofluorometer system (Birmingham, NJ). The Tb^{3+} emission fluorescence peak at 543 nm upon tyrosine excitation at 280 nm was quantified as previously described (Wallace et al., 1982).

5.8 Phosphorylation of calmodulin

Phosphorylation of CaM was carried out at 37 °C for 30 min in a total volume of 0.1 ml in a buffer containing 15 mM Hepes-NaOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1.2 µM histone, 1.2 µM CaM (different species), and 0.02 µg of recombinant c-Src. The reaction was started upon addition of 2 mM ATP and stopped upon addition of Laemmli buffer and heating the sample at 100 °C for 5 min. Tyrosine-phosphorylated CaM and auto-phosphorylated c-Src were detected by standard Western blot technique using the anti-phospho-tyrosine 4G10 antibody. Membrane segments were stripped at 50 °C for 45 min in a buffer containing 2 % (w/v) SDS, 60 mM Tris-HCl (pH 6.8) and 0.1 % (v/v) β-mercaptoethanol, extensively washed with water, incubated in TBST buffer (0.1 % (w/v) Tween-20, 100 mM Tris-HCl (pH 8.8), 500 mM NaCl and 0.25 mM KCl) for 30 min and reprobed with either anti-Src or anti-CaM antibodies as loading controls. Alternatively, when larger amounts of phospho-(Y)-CaM was required to assay its action on target proteins, CaM (1 mg) was phosphorylated in a 1 ml reaction mixture containing 400 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM ATP, 2 mM EGTA, 1 mM DTT, 1 mg histone, and 8 µg c-Src at 37 ºC overnight as described (Corti et al., 1999). Tyrosine-phosphorylated CaM, free of non-phosphorylated CaM, was obtained by affinity-purification using an immobilized anti-phospho-tyrosine antibody as previously described (Palomo-Jiménez et al., 1999).

5.9 Phosphodiesterase assay

The CaM-dependent cyclic nucleotide PDE1 was assayed as previously described (Palomo-Jiménez et al., 1999) at 37 °C for 15 min in 250 µl of a medium containing 50 mM imidazole-HCl (pH 7.5), 10 mM Hepes-NaOH (pH 7.4), 0.2 M NaCl, 0.4 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.4 mM EGTA, 0.5 mM $CaCl_2$ (0.1 mM free Ca^{2+}), 2.5 mM cAMP, 0.01 units of cyclic nucleotide PDE1, 2 units of 5'-nucleotidase, and the concentrations of the different CaM species indicated in the legend to the figures. One unit of cyclic nucleotide PDE1 transforms 1 µmol of cAMP to AMP per min at 30 °C and pH 7.5. One unit of 5'-nucleotidase releases 1 µmol of inorganic phosphate from AMP per min at 37 °C and pH 9. The inorganic phosphate released from AMP was determined colorimetrically based in a described method (Fiske and Subbarow, 1925).

5.10 Isothermal titration calorimetry

Determination of the kinetics parameters of PDE1 in the absence and presence of wild type CaM and CaM(Y99D/Y138D) were done in a VP-ITC instrument (GE-Healthcare). The reaction cell (1.4619 ml) was filled with degassed protein solutions and equilibrated at 37 ºC. Stirring speed was 307 rpm and thermal power was

registered every 2 seconds with an instrumental reference power of 20 µcal/s. Enzyme reaction rates were determined by measuring the change in the instrumental power supplied to the sample cell after addition of the substrate (Todd and Gomez, 2001). At least three independent measurements were carried out in each experimental condition. Serial injections of 4.7 mM cAMP were made every 2 min into the sample cell loaded with 0.01-0.04 U of PDE1 in the absence or presence of wild type CaM or the Y99D/Y138D mutant (3.8 µM) in the same reaction buffer as described in 5.9. To avoid distortions associated with dilution events, the power change associated with substrate addition at injection *i* was averaged over the 30 s immediately before the injection *i*+1. Reaction rates under steady-state conditions were then calculated dividing the power change for each injection, $(dQ/dt)_i$, by the cell volume and the apparent reaction enthalpy (ΔH_{app}) using the software provided by the manufacturer (Origin ITC 7.0). The complete enthalpy of the reaction was determined in separate experiments where 15 µl of 4.7 mM cAMP were injected into the cell filled with a solution containing 0.1 units PDE1 and 3.8 µM CaM wild type and the hydrolysis was monitored until initial baseline was recovered. Integration of the area under the peak gave the total heat of hydrolysis that was corrected by the heat of cAMP dilution, measured independently by injecting the substrate solution in the sample cell loaded with buffer. Very similar values of ΔH_{app} (-19.9 ± 0.5 kcal/mol) were obtained with subsequent injections, indicative of no product inhibition.

5.11 Nitric oxide synthase assay

Recombinant eNOS was assayed determining the accumulation of nitrite as by-product of NO oxidation using a nitrate reductase/colorimetric assay kit (Oxford Biomedical Research, UK) exogenously supplemented with 25 µM FAD, 25 µM FMN, 12 µM tetrahydrobiopterin and the concentrations of the different CaM species indicated in the legends to the figures following the recommendation of the supplier. One unit of eNOS produces 1 nmol/min of NO from arginine at 37 ºC and pH 7.4.

5.12 Determination of free Ca^{2+} concentrations

The concentrations of free Ca^{2+} in the PDE1 and eNOS assay systems were determined using established algorithms of the Maxchelator program. This program is freely available at http://maxchelator.stamford.edu.

5.13 Cell culture

Human epidermoid carcinoma A431 cells, human cervix adenocarcinoma HeLa cells, human lung carcinoma A549 cells, human breast adenocarcinoma SK-BR-3 cells and mouse EGFR-T17 fibroblasts, a stably transfected cell line overexpressing the

human EGFR, were grown in DMEM supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine and 40 µg/ml gentamicin in a humidified atmosphere of 5 % (v/v) CO_2 in air at 37°C. In case of activation of the EGFR or c-Src kinases the cells were maintained overnight or four hours in a fetal bovine serum-free medium before performing the experiments. DT40wt/EGFR cells clone G5, a chicken pre-B lymphoma cell line stably transfected with the human EGFR; and the ET1–55/EGFR clone H8, a conditional CaM-KO cell line stably transfected with the human EGFR, where the expression of recombinant rat CaM is negatively controlled by a tetracycline response element (Tet-Off system) (Panina et al., 2012), were grown in RPMI supplemented with 1 % (v/v) chicken serum, 10 % (v/v) fetal bovine serum, 2 mM L-glutamine and 40 µg/ml gentamicin. When CaM down-regulation was performed, the ET1-55/EGFR clone H8 and DT40wt EGFR clone G5 cells (negative control) were treated with 1 µg/ml tetracycline for the times indicated in the figure legends.

5.14 Preparation of cell membrane fraction

A431 cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Na/K-phosphate, pH 7.4), gently scraped from the plates, harvested by centrifugation, and lysed in 3 ml of an ice-cold hypotonic buffer containing 15 mM Hepes-Na (pH 7.4), 1 mM EGTA, and a cocktail of protease inhibitors (0.5 mM AEBSF, 0.4 µM aprotinin, 25 µM bestatin, 7.5 µM E-64, 10 µM leupeptin, 5 µM pepstatin A, and freshly prepared 0.6 mM PMSF). The lysate was incubated 10 min on ice and centrifuged at 130,000 g for 30 min at 4 °C. The supernatant was discarded, and the pellet resuspended in the same buffer and centrifuged as above. This pellet corresponding to the membrane fraction was washed with the same buffer but without EGTA, centrifuged again, and resuspended in 3 ml of 25 mM Hepes-Na (pH 7.4) containing the protease inhibitors.

5.15 Electrophoresis and Western blots

Proteins were separated by slab SDS-PAGE in 5-20 % (w/v) linear gradient of polyacrylamide and 0.1 % (w/v) SDS at pH 8.3 according to a described method (Laemmli, 1970) at 6 mA during 16-17 h. After processing by SDS-PAGE, proteins were electro-transferred from the gel to a PVDF membrane or nitrocellulose in case we were testing for CaM for 2 h at 300 mA in a buffer containing 48 mM Tris-base, 36.6 mM L-glycine, 0.04 % (w/v) SDS and 20 % (v/v) methanol (TGSM buffer); fixed with 0.8 % (v/v) glutaraldehyde in T-TBS buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1 % (v/v) Tween-20 for 10 minutes and transiently stained with 0.1 % Fast Green FCF (w/v) FCF, 50 % (v/v) methanol, 10 % (v/v) acetic acid to ascertain the regularity of the transfer procedure. The membranes were blocked with 5 % (w/v) bovine serum

albumin, 3 or 5 % (w/v) fat-free powdered milk, as recommended by the antibodies supplier, in T-TBS or T-PBS buffer. This was followed by probing over-night at 4 ºC using a 1/2000 dilution of the corresponding primary antibody, and thereafter for 1 h at room temperature using a 1/5000 dilution of the appropriate secondary anti-IgG or anti-IgM antibodies coupled to horseradish peroxidase. The bands were visualized upon development with the ECL reagents. The intensity of the bands was quantified with a computer-assisted scanning densitometry using the ImageJ (1.47v) program.

5.16 *In vitro* EGFR phosphorylation assays

EGFR was assayed in a reaction mixture containing the detergent-solubilized receptor from a membrane fraction of A431 cells, 15 mM Hepes-NaO (pH 7.4), 2 mM $MgCl_2$, and 2 mM EGTA or 100 µM $CaCl_2$ were added depending on the condition tested, and 1 µg of each CaM species. The reaction mixture was then incubated on ice for 30 min in the absence or presence of 1 µM EGF. Thereafter, the reaction was started upon addition of 2 mM ATP at 37 °C for 5 min and stopped with the addition of loading buffer in a final volume of 50 or 100 µl. The samples were boiled at 100 °C for 5 min. centrifuged and analyze by SDS-PAGE and Western blot as detailed above. In case of using recombinant $EGFR_{cyt}$ for the *in vitro* phosphorylation of OGT, the reaction took place as follows: 250 ng of the recombinant $EGFR_{cyt}$ were assay in a buffer containing 15 mM Hepes-NaOH (pH 7.4), 5 mM $MgCl_2$, in the absence or presence of 500 ng ncOGT and in the absence or presence of 5 mM ATP. The reaction mixture was then incubated for 2 hours at 37 °C. The reaction was stopped by the addition of loading buffer, boiled for 5 min and processes by SDS-PAGE and Western blot as described above.

5.17 Activation of EGFR in living cells

Cells were grown to confluence in 6-well culture dishes containing 2 ml of DMEM supplemented with 10 % (v/v) FBS overnight, and deprived of FBS for 4 hours as mention above. In order to study the phosphorylation of EGFR induced by the EGF the following was done: 10 nM EGF was added to cells in 2 ml of serum-free culture medium and the incubation was continued for different periods of time (0, 2, 5, 10, 15 and 30 min). The reaction was arrested upon addition of ice-cold 10 % (w/v) trichloroacetic acid (TCA). The cells were scraped from the culture dish and centrifuged at 16,060 g for 10 min. The supernatant was discarded and the proteins were resuspended in Laemmli buffer, boiled at 100°C for 10 min and centrifuged again for 1 min. The tyrosine phosphorylation level of EGFR and proper loading controls were determined by SDS-PAGE and Western blot analysis using an anti-phospho-tyrosine

and anti-GAPDH or anti-tubulin-α antibodies, respectively.

5.18 CaM-affinity chromatography and immobilized-CaM pull-down of Src

Cell membrane-anchored Src from A431 cells was detached from the membrane fraction upon incubation with 1 % (w/v) Triton X-100 and 5 % (w/v) glycerol at 0 °C for 10 min. The sample was centrifuged at 130,000 g for 30 min and the supernatant was used to isolate Src by CaM-affinity chromatography or pull-down using immobilized CaM. The isolation of Src by Ca^{2+}-dependent CaM-affinity chromatography was carried out loading the solubilized membrane fraction in a small column (1-2 ml bed volume) of CaM-Sepharose 4B equilibrated with a buffer containing 25 mM Hepes-NaOH (pH 7.4), 1 % (w/v) Triton X-100, 5 % (w/v) glycerol, 0.1 mM $CaCl_2$, and the protease inhibitors cocktail described above (Ca^{2+}-buffer). The column was washed with 25 volumes of the Ca^{2+}-buffer, and Src was eluted using the same buffer but containing 1 mM EGTA instead of $CaCl_2$ (EGTA-buffer). Proteins in the eluted fractions (0.6 ml) were precipitated with 10 % (w/v) TCA and processed by SDS-PAGE and Western blot for Src identification. The pull-down of Src was done using a slurry of CaM-Sepharose 4B beads (200 µl) in a buffer containing 25 mM Na-Hepes (pH 7.4), 1 % (w/v) Triton X-100 and 5 % (w/v) glycerol supplemented either with 0.1 mM $CaCl_2$ or 1 mM EGTA. We used naked Sepharose 4B beads as negative binding control. The beads were washed 10 times with the corresponding Ca^{2+} or EGTA buffers before processing the samples by SDS-PAGE and Western blot for Src identification as described above.

5.19 Co-immunoprecipitation assays

Immunoprecipitation of Src, EGFR and OGT from A431 cells (2 mg protein) was performed using the Pierce™ Classic Magnetic IP/Co-IP kit, and anti-Src, anti-EGFR or anti-OGT antibodies and protein-A/G following the manufacture instructions. The samples were processed for SDS-PAGE and Western blot using the appropriate antibodies as described above.

5.20 Src phosphorylation assays

The auto-phosphorylation of recombinant c-Src (0.1 µg) was assayed at 37 ºC in a medium containing 15 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 1.2 mM $CaCl_2$ (when added), and 2.3 µM CaM wild type, or the phospho-mimetic CaM(Y99D/Y138D) and CaM(Y99E/Y138E) mutants, when added. The reaction was started upon addition of 2 mM ATP and stopped by the addition of Laemmli buffer and boiled for 5 min. The samples were subjected to Western blot and probed with an anti-phospho-tyrosine antibody (4G10) or an anti-phospho-Y416-Src specific antibody to

detect active phosphorylated Src, and an anti-Src (total) antibody and/or GAPDH antibody as loading controls.

5.21 Src activation assays in living cells

In the case of A431 cells, Src was activated either upon ligand-dependent activation of the EGFR, as an upstream signaling component of c-Src (Sato et al., 2001), adding 10 nM EGF during 2 min; and in the case of both A431 and SK-BR-3 cells, upon induction of oxidative stress by adding 1 mM H_2O_2 for 15 min, as described (Basuroy et al., 2010). The reaction was arrested upon addition of ice-cold 1 % (v/v) TCA, and the samples were processed by SDS-PAGE and Western blot. The PVDF membranes were probed with anti-phospho-tyrosine (4G10) or anti-phospho-Y416-Src specific antibodies to detect active phosphorylated Src, anti-phospho-tyrosine antibody to detect activated EGFR, and anti-Src (total), anti-EGFR (total) and/or anti-GAPDH antibodies as loading controls.

5.22 *O*-GlcNAc detection in EGFR

EGFR was immunoprecipitated from A431 cells using the Pierce Classic Magnetic IP/Co-IP kit following the manufacturer instructions. Briefly, a lysate of A431 cells (1-2 mg proteins) were incubated with anti-EGFR antibody (1:500 dilution) overnight at 4 ºC. The lysate containing the antigen-antibody complex was mixed with the magnetic beads and incubated for 1 hour at room temperature. The beads were extensively washed and the EGFR was released with the low pH (pH 2) elution buffer, resolved in SDS-PAGE and subjected to Western blot using appropriate anti-*O*-GlcNAc antibodies (RL2 or CTD110.6). Conversely, a lysate of A431 cells (1-2 mg proteins) were incubated as above with an anti-*O*-GlcNAc antibody (RL2) and the immunoprecipitated *O*-GlcNAcylated proteins were resolved in SDS-PAGE and subjected to Western blot using and anti-EGFR antibody.

5.23 *In vitro* EGFR *O*-GlcNAcylation

Immunoprecipitated EGFR was used for *in vitro* *O*-GlcNAcylation assay using immunoprecipitated OGT, both immunoprecipitated from A431 cells. The *O*-GlcNAcylation reaction took place in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 12.5 mM $MgCl_2$ and 1 mM UDP-GlcNAc, and incubated for 2.5 h at 30 ºC. The reaction was stopped by adding Laemmli buffer and boiled for 10 min at 100 ºC, resolved in SDS-PAGE, transferred to a PVDF membrane, and probed with an anti-*O*-GlcNAc antibody (RL2).

5.24 Metabolic protein labeling with azido-GlcNAc

A431 cells were seeded in 150 cm^2 petri dishes and when ~ 70 % confluence was obtained 40 µM GlcNAz was added and incubated for 72 h. The cells were lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 % (v/v) NP-40, 5 % (v/v) glycerol supplemented with 20 µM PUGNAc to prevent deglycosylation and a cocktail of protease inhibitors (0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.4 µM aprotinin, 25 µM bestatin, 7.5 µM [1-[N-[(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane], 10 µM leupeptin, 5 µM pepstatin A, and freshly prepared 0.6 mM PMSF). The lysate was centrifuged at 16,000 g for 10 min and the supernatant used for EGFR immunoprecipitation. Biotinylation of the GlcNAz-labeled proteins was performed using Click-iT®-Protein Reaction Buffer kit following the manufacturer's instructions, the samples were subjected to SDS-PAGE, the proteins transferred to PVDF and overlaid with streptavidin-HRP (1:10,000 dilution).

5.25 *N*-deglycosylation of the EGFR

The immunoprecipitated EGFR was first denatured in a buffer containing 0.5 % (w/v) SDS and 40 mM DTT and boiled for 10 min at 100 °C. Thereafter, 10 % (v/v) NP-40, 50 mM sodium phosphate (pH 7.5) and 1,000 units of PNGase F were added, and the mixture was incubated for 2 h at 37 ºC. One unit of PNGase F is defined as the amount of enzyme required to remove > 95 % of the carbohydrates from 10 µg of denatured RNase B in 1 hour at 37 °C. The reaction was stopped by the addition of Laemmli buffer and boiled for 10 min at 100 ºC, resolved in SDS-PAGE, transferred to a PVDF membrane and probed with anti-*O*-GlcNAc antibodies (RL2 or CTD110.6). When tunicamycin was used as a deglycosylation agent A431 cells were cultured in complete DMEM supplemented with 1 µg/ml tunicamycin overnight where only newly synthetized EGFR molecules are expected to be fully N-deglycosylated. Higher concentrations of tunicamycin to attain more N-deglycosylated EGFR molecules was not possible as more drastic treatments result in extensive cell death.

5.26 *O*-deglycosylation of the EGFR

The immunoprecipitated EGFR was first denatured in a buffer containing 0.5 % (w/v) SDS and 40 mM DTT and boiled for 10 min at 100 °C. Thereafter, 10 % (v/v) NP-40, 50 mM sodium phosphate (pH 7.5) and 200,000 units of *O*-glycosidase and 100 units of neuraminidase were added, and the mixture was incubated for 2 h at 37 ºC. One unit of *O*-glycosidase is defined as the amount of enzyme required to remove 0.68 nmol of *O*-linked disaccharide from 5 mg of neuraminidase digested, non-denatured fetuin in 1 hour at 37°C in a total reaction volume of 100 µl. The reaction was stopped

by the addition of Laemmli buffer and boiled for 10 min at 100 ºC, resolved in SDS-PAGE, transferred to a nitrocellulose membrane and overlaid with the lectins WGA and PNA.

5.27 Effect of OGT and OGA inhibitors on EGFR *O*-GlcNAcylation

A431 cells were grown to confluence in 6-well culture dishes containing 2 ml of DMEM supplemented with 10 % (v/v) FBS overnight in the absence and presence of the OGT inhibitor BADGP (2 mM), or the OGA inhibitor Thiamet G (20 µM). Thereafter, the EGFR was immunoprecipitated from a cell lysate using an anti-EGFR antibody and the immunocomplex was processed by SDS-PAGE and Western blot using an anti-*O*-GlcNAc antibody (RL2). The effect of 2 mM BADGP and increasing concentrations (0.5-20 µM) of Thiamet G was also tested on total proteins *O*-GlcNAcylation level from an A431 cell lysate as positive control.

5.28 Expression and purification of recombinant ncOGT

His-tagged full-length human OGT (ncOGT) recombinant protein was expressed and purified as previously described (Gross et al., 2005). Briefly, the plasmid containing the sequence encoding the ncOGT was expressed in *E. coli* BL21(DE3)pLysS cells. Single colonies grown in solid medium in the presence of 100 µg/ml kanamycin were collected and seeded in 5 ml of Luria´s broth containing the same concentration of kanamycin. Larger cultures (500 ml) were seeded with the pre-culture and grown in the same conditions until they reached an $OD_{600\ nm}$ = 1-1.2. The expression of the recombinant protein was induced with 0.1-0.2 mM IPTG overnight at 16 ºC. The purification of the His-tagged ncOGT was performed using the BugBuster® Ni-NTA His•Bind® Purification kit according to the manufacturer instructions.

5.29 Lectin overlay

The lectin overlay was performed according to the manufacturer protocol with small modifications. Briefly, the detergent-solubilized EGFR was immunoprecipitated from A431 cells and treated with PNGase F, *O*-glycosidase, and Neuraminidase separated in 5-20 % polyacrylamide gradient gel and transferred to nitrocellulose membranes as described above. The membranes were then washed in PBS (pH 7.5) and blocked for two minutes with PBS (pH 7.5) with 0.25 % (v/v) Tween-20 and rinsed with PBS (pH 7.5), 0.05 % (v/v) Tween-20, twice. Afterwards the membranes were overlaid with 1 µg/ml biotin-labeled WGA or PNA for two hours at 20 °C in a buffer containing PBS (pH 7.5), 0.1 % (v/v) Tween-20, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$. Then the membranes were extensively washed with PBS (pH 7.5) 0.1 % (v/v) Tween-20 and incubated with streptavidin-HRP (1:10000) for 45 min in PBS (pH 7.5)

0.1 % (v/v) Tween-20, extensively washed with PBS with 0.05 % (v/v) Tween-20 and revealed with ECL.

5.30 Artificial wound-healing assays

Artificial wounds were done with a plastic pipette scratching a monolayer of confluent A431. The wounded monolayers were washed twice with fresh media to remove non-adherent cells before photographs were taken every hour for 72 hours using a Cell Observer system equipped with Zeiss Axiovert 200M Multi-stage Automated microscope with a 4x objective to follow the repopulation of the wounds. The cells were incubated in DMEM containing 1 % (v/v) FBS, in the absence or the presence of 10 nM EGF, 100 µM Thiamet G or 1 mM BADGP.

5.31 Ca^{2+}-dependent CaM-affinity chromatography of OGT

The isolation of recombinant ncOGT by Ca^{2+}-dependent CaM-affinity chromatography was carried out loading the purified recombinant ncOGT in a small column (1-2 ml bed volume) of CaM-Sepharose 4B equilibrated with a buffer containing 25 mM Hepes-NaOH (pH 7.4), 1 % (w/v) Triton X-100, 5 % (w/v) glycerol, 0.1 mM $CaCl_2$, and the protease inhibitors cocktail described above (Ca^{2+}-buffer). The column was washed with 20 volumes of the Ca^{2+}-buffer, and ncOGT was eluted using the same buffer but containing 1 mM EGTA instead of $CaCl_2$ (EGTA-buffer). Proteins in the eluted fractions were precipitated with 10 % (w/v) TCA and processed by SDS-PAGE and Western blot for OGT identification using an anti-OGT antibody.

5.32 Statistical analysis

The paired Student's t and the two-way ANOVA tests were performed using the Microsoft Excel (Microsoft Co., Redmon, WA) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA) software programs. Data were expressed as the mean ± SEM or SD/range and differences were considered significant at $p \leq 0.05$ as indicated in the legends to the figures.

6. RESULTS

As CaM can be phosphorylated (Benaim and Villalobo, 2002), and in order to better understand its physiological functions in systems relevant for tumor cell biology, we decided to study the functionality of phospho-(Y)-CaM using a set of recombinant phospho-(Y)-mimetic CaM mutants by substituting either one or the two tyrosine residues to aspartic acid (Asp, D) or glutamic acid (Glu, E). Then we characterized their physicochemical properties and tested *in vitro* their action on the activity of several CaM-dependent enzymes: the 3',5'-cyclic nucleotide PDE1 (EC 3.1.4.17), eNOS (EC 1.14.13.39), c-Src (EC 2.7.10.2) and EGFR (EC 2.7.10.1).

6.1. Expression and purification of the phospho-(Y)-mimetic CaM mutants

In order to confirm if the generated mutants preserve the general structural and functional properties of wild type CaM and modulate CaM-dependent systems such as PDE1, eNOS, EGFR, and c-Src we performed an extensive physicochemical characterization of the generated CaM mutant species. The first step was to transform *E. coli* BL21(DE3)pLysS with the vectors containing the sequence of the whole panel of mutants: CaM(Y99D), CaM(Y138D), CaM(Y99D/Y138D), CaM(Y99E), CaM(Y138E) and CaM(Y99E/Y138E) and purify significant amount of each species. The expression of wild type and the different CaM mutants in *E.coli* BL21(DE3)pLysS was very efficient and their purification yielded an average ± SEM (n = 12) 13 ± 2 mg CaM per 500 ml of bacterial culture. Figure 8A shows an example of the expression of the mutants CaM(Y99E), CaM(Y138E), CaM(Y99E)/Y138E, CaM(Y99D), CaM(Y138D), and CaM(Y99D/Y138D) induced by IPTG addition, and the material obtained in the supernatant after heating the extracted proteins at 95 ºC for 5 min. It can be seen that CaM is the majoritarian heat-resistant protein. After Ca^{2+}-dependent phenyl-Sepharose chromatography all purified CaM species were homogeneous in SDS-PAGE (Figure 8B). The CaM variants presented a single band (~ 18 kDa) when electrophoretically separated in the presence of EGTA (absence of Ca^{2+}). In the presence of Ca^{2+}, however, wild type CaM and both CaM(Y99D) and CaM(Y99E) mutants presented the typical Ca^{2+}-induced electrophoretic mobility shift (Burgess et al., 1980), yielding a major band at ~ 15 kDa and a minor one with lower mobility. The electrophoretic mobility shift was less apparent in the single mutants CaM(Y138D) and CaM(Y138E), and in the double mutants CaM(Y99D/Y138D) and CaM(Y99E/Y138E) (Figure 8B).

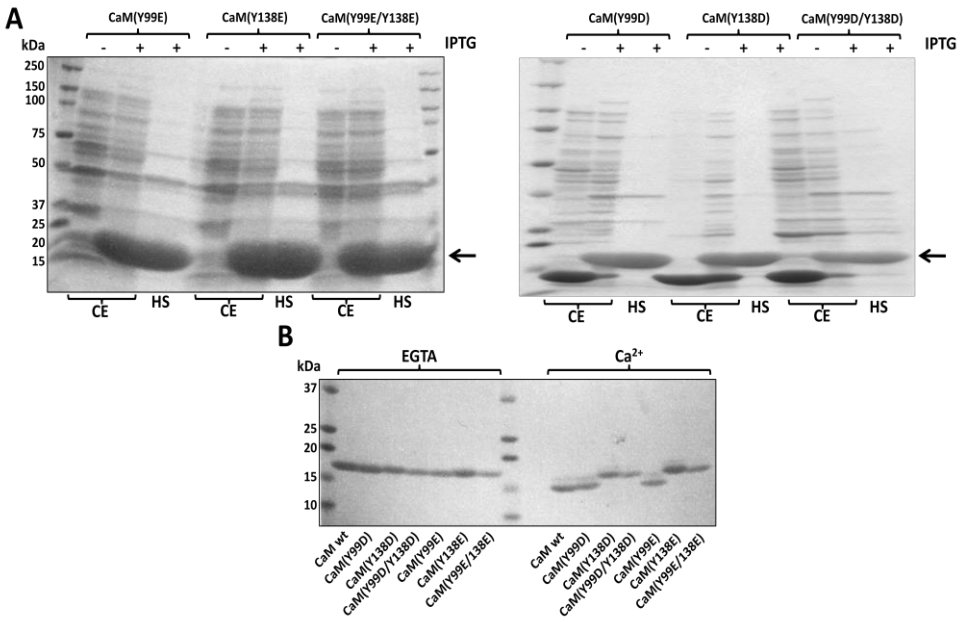

Figure 8: Expression and purification of the different CaM species. (*A*) The figure shows the pattern of proteins of the bacterial cell extract (CE) and the corresponding heated supernatant (HS) of CaM Y/E mutant species (*left panel*) and CaM Y/D mutant species (*right panel*) in the absence and presence of 0.5 mM IPTG for 4 h as described in Materials and Methods. (*B*) The different recombinant CaM species (~ 1-2 µg) purified as described in Materials and Methods were separated by SDS-PAGE in the presence of 5 mM EGTA or 1 mM $CaCl_2$ to observe the Ca^{2+}-induced mobility shift.

6.1.1 UV absorption spectra of the phospho-(Y)-mimetic CaM mutants

Since CaM has no tryptophan (W, Trp) to contribute to the UV-spectrum of the protein in the range of 280 nm, the tyrosine (Y, Tyr) absorption peak at 276 nm is very well defined, thus allowing us to detect the changes taking place in that region due to the subsequent removal of one or two Tyr residues. The UV absorption spectra (240-340 nm) of wild type CaM and the whole panel of Y/D and Y/E mutants are shown in Figure 9. As expected wild type CaM showed the characteristic absorption peaks at 252, 258, 265, 269 and 276 nm (Wolff et al., 1977), and the latest one due to the presence of tyrosine residues. As expected, the single Y/D and Y/E CaM mutants presented a significant reduction of the 276 nm peak, while this peak totally disappeared in the double mutants CaM(Y99D/Y138D) and CaM(Y99E/Y138E). The Y99D and Y99E substitutions presented a slightly higher decrease in the 270-285 nm region of the absorption spectrum than identical substitutions at Y138, pointing out a higher contribution of Y99 to the spectrum of wild type CaM. Moreover, no significant spectral differences between the respective Y/D and Y/E CaM mutants were found.

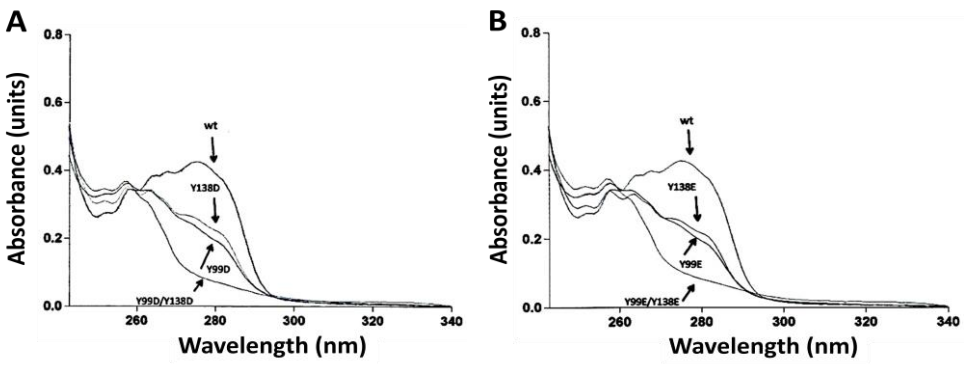

Figure 9: Absorption spectra of the different CaM species. The plots show UV-light absorption spectra of recombinant wild type (wt) and the indicated Y/D (A) and Y/E (B) CaM mutants (2 mg/ml) purified as described in Materials and Methods and dialyzed against 20 mM Tris-HCl (pH 7.5).

6.1.2 Circular dichroism studies of the phospho-(Y)-mimetic CaM mutants

Circular dichroism (CD) is a very powerful tool to study conformational features of proteins in solution (Kelly et al., 2005). In the absence of Ca^{2+}, the far-UV (200-260 nm) CD spectra of wild type and the full set of CaM mutants showed the characteristic negative maxima at 208 and 222 nm (Figure 10), typical of proteins with a high percentage of α-helical content. Ca^{2+} binding induces further deepening of the spectra due to an increase in the amount of α-helical structure (Martin & Bayley, 1986).

However, the ratio of molar ellipticity at 208 and 222 nm, a sensitive indicator of possible alterations in interactions between neighboring helices (Sun et al., 2001, Fasman, 1996), was slightly higher for the mutants (Table 3), pointing to subtle differences in helix packing compared to wild type CaM. For all the samples, there was a significant, mutant-specific, deepening of the far-UV signals in the presence of Ca^{2+} that may reflect a differential increase in α-helical content and/or reorientation of existing α-helices (Wang et al., 2011, Protasevich et al., 1997). Also, both CaM(Y99D/Y138D) and CaM(Y99E/Y138E) mutants presented a slight blue-shift of the 208 nm minimum in the absence of Ca^{2+} as compared to wild type CaM (Figure 10).

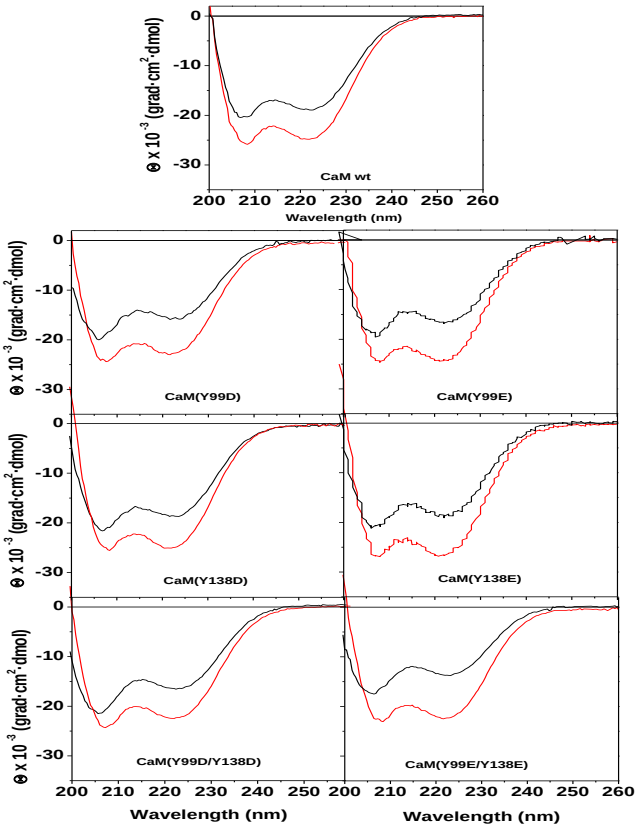

Figure 10: Ca^{2+}-induced changes in the Far-UV CD spectra of the different CaM species. Far-UV CD spectra of wild type (wt) and the Y/D and Y/E CaM mutants (12 μM) were recorded at 20 ºC in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, containing 1 mM EGTA (black lines) or 1 mM $CaCl_2$ (red lines).

As shown in Figure 11A, the ellipticity at 222 nm increased in the presence of Ca^{2+} 22 ± 3 % for wild type CaM, while for the Y138D and Y138E mutants the increase was of 32-35 %, and for the Y99D, Y99E, and double Y/E mutants it reached values of 50 % and above, the only exception being the CaM(Y99D/Y138D) mutant where no significant change was observed. Interestingly, the magnitude of the increase in ellipticity at 222 nm is directly correlated to the value of the $\Theta_{208}/\Theta_{222}$ ratio. On the other hand, percentage of Ca^{2+}-induced increases in ellipticity at 208 nm and variations among CaM species were noticeably smaller, going from 14 % to 29 % (Figure 11B).

As a result, no significant differences in the $\Theta_{208}/\Theta_{222}$ ratio were observed in the presence of Ca^{2+}, again with the only exception of a small change in the CaM(Y99D/Y138D) mutant (Table 1) revealing a similar overall structure of the holo forms. Above all, far-UV data clearly show that all the single and double Y/D and Y/E CaM mutants bind Ca^{2+}.

Sample	$\Theta_{208}/\Theta_{222}$		Θ_{208} Ca^{2+}/ Θ_{208} EGTA	Θ_{222} Ca^{2+}/ Θ_{222} EGTA
	EGTA	Ca^{2+}		
CaM wt	1.09 ± 0.01	1.01 ± 0.01	1.14 ± 0.05	1.22 ± 0.03
CaM(Y99D)	1.20 ± 0.01	1.01 ± 0.01	1.29 ± 0.01	1.52 ± 0.03
CaM(Y99E)	1.17 ± 0.01	1.00 ± 0.01	1.26 ± 0.01	1.48 ± 0.02
CaM(Y138D)	1.12 ± 0.01	1.01 ± 0.01	1.22 ± 0.02	1.35 ± 0.02
CaM(Y138E)	1.14 ± 0.02	1.02 ± 0.01	1.19 ± 0.06	1.32 ± 0.05
CaM(Y99D/Y138D)	1.23 ± 0.03	1.10 ± 0.01	1.15 ± 0.09	1.21 ± 0.05
CaM(Y99E/Y138E)	1.19 ± 0.02	1.03 ± 0.01	1.26 ± 0.03	1.55 ± 0.08

Table 3: $\Theta_{208}/\Theta_{222}$ ellipticity ratio in the absence and presence of Ca^{2+} of the different CaM species.

Ca^{2+}-binding also induces noticeable changes in the near-UV circular dichroism spectrum of wild type CaM (Sun et al., 2001, Wolff et al., 1977, Martin and Bayley, 1986), which is sensitive to the environment of the six phenylalanines and the two tyrosine residues in the molecule. As shown in Figure 12, in the presence of EGTA the spectrum is characterized by two well-defined negative bands at 262 and 269 nm, due to the phenylalanine residues, and a broad negative region centered at 280 nm due to the tyrosine residues.

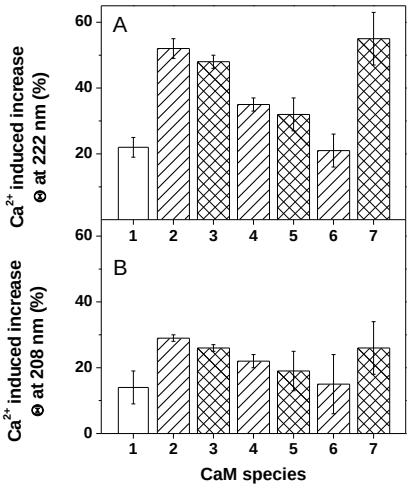

Figure 11: Percentage increase of ellipticity of the different CaM mutants in the presence of Ca^{2+} detected by CD at 222 and 208 nm. The mean ± SEM (n = 3) increase in ellipticity in the far-UV circular dichroism signals at 222 nm (A) and 208 nm (B) of the different CaM species 1: CaM wild type; 2, CaM(Y99D); 3, CaM(Y99E); 4, CaM(Y138D); 5, CaM(Y138E); 6, CaM(Y99D)/Y138D; and 7, CaM(Y99E)/Y138E) is represented as percentage taking as 100 % the ellipticity of each CaM species in the presence of EGTA. Measurements were carried out at 20 ºC in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, containing 1 mM EGTA or 1 mM $CaCl_2$. The concentration of CaM was 12 μM.

Addition of Ca^{2+} results in an increase of these ellipticity signals, what has been interpreted as a change in the environment of both phenylalanine and tyrosine (when present) residues. Having confirmed by far-UV CD experiment that all single and double Y/D and Y/E mutants bind Ca^{2+}, we also examined the near-UV CD spectra of their apo and holo forms. In the absence of Ca^{2+}, the ellipticity around 280 nm of the mutants diminished noticeably or even became positive (Figure 12), confirming a

predominant contribution of the two tyrosine residues to this region in wild type CaM. In addition, there was a significant decrease in the intensity of the bands at 262 and 269 nm compared to wild type CaM, pointing to a partial contribution of both tyrosine residues to these signals. However, addition of Ca^{2+} induced different changes in the spectra depending on the mutant. While the Y99D/E mutants showed a small decrease in positive ellipticity at 280 nm, for the respective Y138 mutants the ellipticity change becames almost zero (Figure 12). Differences related to the 262/269 nm bands were even more apparent.

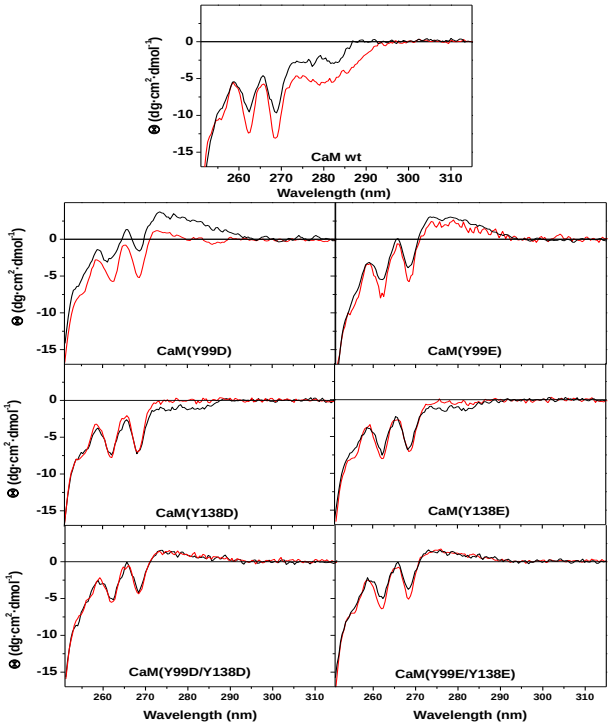

Figure 12: Ca^{2+}-induced changes in the near-UV circular dichroism spectra of the different CaM species. Near-UV CD spectra for wild type (*wt*) and the single and double Y/D and Y/E CaM mutants (118 µM) were recorded at 20 ºC in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, containing 1 mM EGTA (black lines) or 1 mM $CaCl_2$ (red lines). Ellipticity values were normalized per mole of residue.

There was a clear increase in ellipticity for the Y99D/E mutants upon Ca^{2+} binding, while little or no change was observed for the Y138D/E and double mutants (Figure 12), clearly revealing that the Ca^{2+}-induced alterations observed for wild type CaM in this region are mostly due to changes in the environment of Y138. This conclusion fully explains the small effect of Ca^{2+} on the near-UV CD spectrum of the separate CaM N-terminal half (residues 1-77) previously reported (Martin and Bayley, 1986), a result considered surprising by the authors in view of the similar distribution of phenylalanine residues in the N- and C-terminal halves of the protein. Indeed, the spectra with/without

Ca^{2+} of the Y138D/E and double CaM mutants (Figure 12) are very similar to those published for the N-terminal half of CaM (Martin and Bayley, 1986).

It is known that Ca^{2+} binding induces a remarkable increase in the thermal stability of wild type CaM (Wang et al., 2011, Brzeska et al., 1983). In order to test whether the same was true for the single and double Y/D and Y/E mutants, we carried out thermal denaturation experiments by measuring changes in ellipticity at 222 nm (Supplementary Figure 1S) at increasing temperature. Figure 13 shows a quasi-sigmoidal decrease in ellipticity of wild type and all CaM mutants in the absence of Ca^{2+}, indicating the occurrence of at least one intermediate state, as previously described for wild type CaM (Sun et al., 2001, Wang et al., 2011). In the case of the CaM Y138D/E mutants, the temperature-dependent unfolding was clearly biphasic, showing a notable deviation of sigmoidicity at temperatures below 50 ºC.

Although fitting the data to a two- or three-state model did not yield acceptable clear-cut information, it is evident from the denaturation profiles that a major thermal unfolding for the different CaM species occurred at ~ 55 ºC, all of them being completely unfolded above 70 ºC. In addition, lower temperature transitions and/or discontinuities, also reported for wild type CaM (Brzeska et al., 1983, Martin and Bayley, 1986, Kilhoffer et al., 1981, Gangola and Pant, 1983) were observed in the ~ 20-40 ºC region, both in the absence and presence of Ca^{2+}. The binding of Ca^{2+} had a profound impact on the stability of wild type CaM and the mutants above 40 ºC. Therefore, the major unfolding transition occurring at ~ 55 ºC in the presence of EGTA was not observed when Ca^{2+} is present, and more than 54 % of the initial ellipticity signal was still preserved at 90 ºC (Figure 13), confirming that Ca^{2+} binding to the single and double Y/D and Y/E CaM mutants also causes remarkable thermal stabilization.

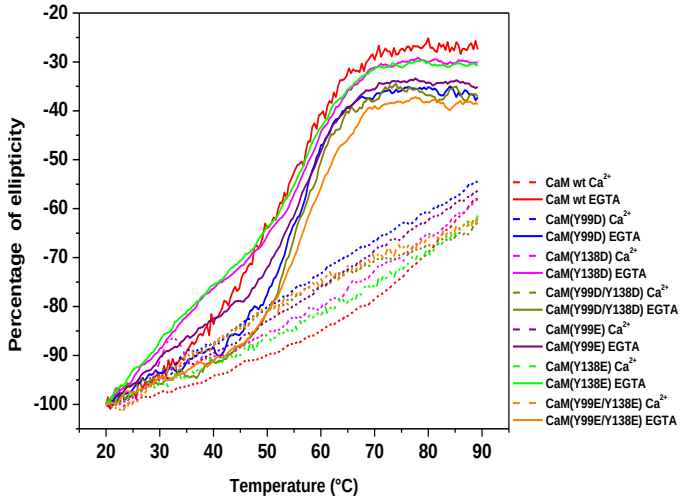

Figure 13: Thermal stability of the different CaM mutants in the absence and presence of Ca^{2+}. The plots present the decrease in the CD signal at 222 nm, expressed as percentage of the ellipticity at 20 ºC, for wild type (*wt*) and the single and double Y/D and Y/E CaM mutants (12 µM) in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, in the presence of 1 mM EGTA (*solid lines*) and 1 mM $CaCl_2$ (*dashed lines*) as described in Materials and Methods.

6.1.3 Fluorescence studies of the phospho-(Y)-mimetic CaM mutants

We also studied the fluorescence energy transfer from excited tyrosine residues to Tb^{3+}, a surrogate ion that binds CaM at the Ca^{2+}-binding sites, in wild type CaM and the different CaM mutant species by measuring the fluorescence emitted by Tb^{3+} in the 535-550 nm region upon exciting at 280 nm the tyrosine residues (when present) located at Ca^{2+}-binding sites III and IV of CaM. The spectrum obtained for wild type CaM presented a distinctive emission peak at 543 nm upon addition of increasing concentrations of Tb^{3+} to the medium (Figure 14A). Interestingly, the absence of Y99 induced an almost complete loss of the Tb^{3+} emission peak at 543 nm, whereas the absence of Y138 did not (Figure 14A). A similar behavior was observed for the Y/D and Y/E mutants. The Tb^{3+} titration curves shown in Figures 15B and 15C demonstrated that the apparent binding affinity of the Tb^{3+} ion to wild type CaM was slightly higher (K'_d ~ 20 μM) than for the CaM(Y138D) or CaM(Y138E) mutants (K'_d ~ 50 and 75 μM, respectively). Only trace fluorescence emission of Tb^{3+} was detected in the CaM Y99D/E mutants, and no significant fluorescence was detected in the double Y/D or Y/E mutants.

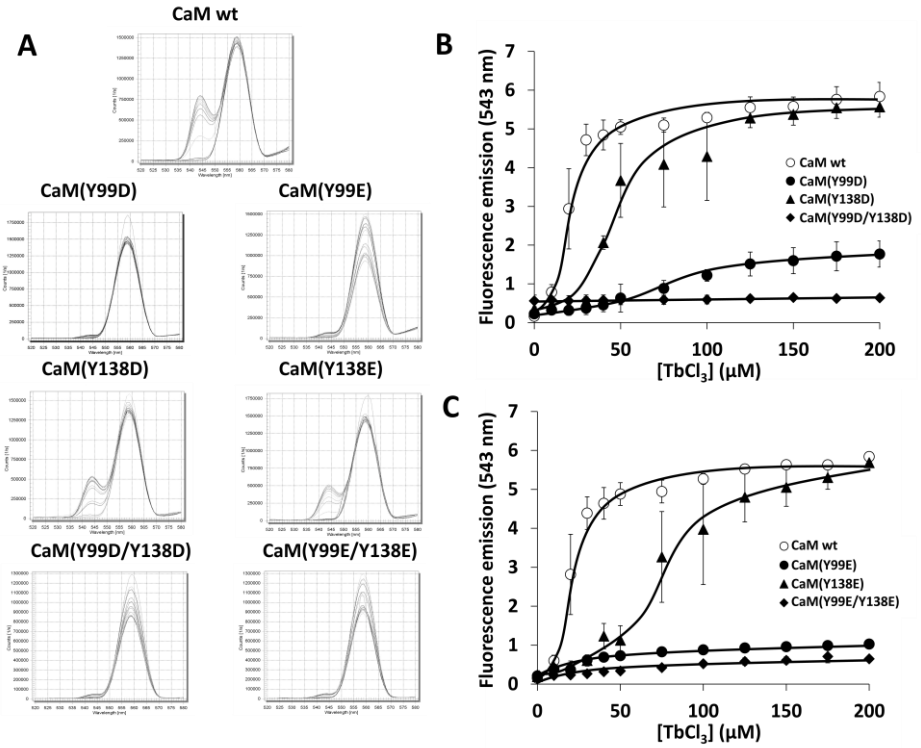

Figure 14: Tb^{3+}-induced fluorescence emission spectra of the different CaM species. (A) The fluorescence emission spectra (520-580 nm) of wild type (*wt*) CaM and the indicated CaM mutants (10 μM) was recorded in a buffer containing 10 mM Pipes-HCl (pH 6.5) and 100 mM KCl in the absence and presence of increasing concentrations of $TbCl_3$ (10-200 μM) as described in Materials an Methods. (B, C) The plots present the emission fluorescence at 543 nm of wild type (*wt*) CaM and the indicated CaM mutants (10 μM) at increasing concentrations of $TbCl_3$ in the conditions described in Materials and Methods.

6.2 Biological activity of the phospho-(Y)-mimetic CaM mutants

We first tested the capacity of c-Src to phosphorylate the different CaM species. Figure 15 shows that both CaM(Y99D) and CaM(Y99E) were phosphorylated *in vitro* by recombinant c-Src with similar efficiency than wild type CaM. However, the CaM Y138D/E mutants were phosphorylated with far lower efficiency than wild type CaM, suggesting that Y138 is preferrebly phosphorylated by the kinase. As expected, no phosphorylation of the double mutants was detected.

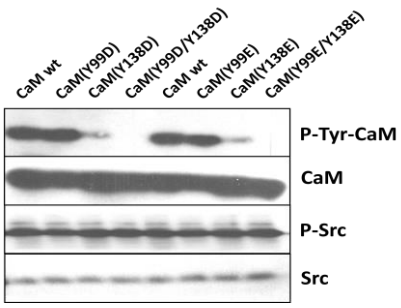

Figure 15: Phosphorylation of different CaM species by recombinant c-Src. The different CaM species (2 µg) were assayed for phosphorylation by recombinant c-Src as described in Materials and Methods. The samples were probed with an anti-phospho-tyrosine antibody to detect the tyrosine-phosphorylated CaM species (*P-(Y)-CaM*) and auto-phosphorylated c-Src (*P-Src*). The membranes were striped and reprobed with anti-CaM and anti-Src antibodies as loading controls.

6.2.1 Effect of the phospho-(Y)-mimetic CaM mutants on PDE1 activity

We next tested the effect of purified phospho-(Y)-CaM free of non-phosphorylated CaM on the CaM-dependent enzyme cyclic nucleotide PDE1. Figure 16A shows the slightly lower activatory effect of phospho-(Y)-CaM compared to non-phosphorylated CaM.

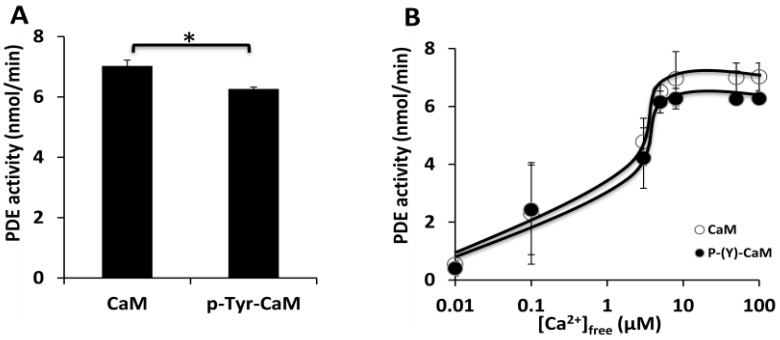

Figure 16: Effect of phospho-(Y)-CaM and non-phosphorylated CaM on the PDE1 activity. (A) The plot presents the average ± SEM PDE1 activity in the presence non-phosphorylated CaM or phospho-(Y)-CaM (0.97 µM) in three independent experiments (* p = 0.03 using the Student's t test) as described in Materials and Methods. (B) The plot presents the average ± range activity of PDE1 assayed in the presence of non-phosphorylated CaM (open symbols) and phospho-(Y)-CaM (filled symbols) (0.97 µM) in two independent experiments assayed at increasing concentrations of free Ca^{2+} as described in Materials and Methods.

In addition, an assay performed at increasing concentrations of free Ca^{2+} in the presence of phospho-(Y)-CaM and non-phosphorylated CaM showed no significant

differences in the apparent affinity for free Ca^{2+} and at saturating concentrations of Ca^{2+} a small decrease (~ 10 %) of PDE1 activity in the presence of phospho-(Y)-CaM compared to non-phosphorylated CaM was observed (Figure 16B).

We then tested the effect of the full set of mutants on the CaM-dependent activation of PDE1. Figure 17 shows that all the Y/D and Y/E CaM mutants were able to activate PDE1 in the presence of Ca^{2+} as it does wild type CaM. However, the CaM-dependent activity attained with CaM(Y99D/Y138D) was consistently lower (~ 20 %) than with the other CaM species, including the CaM(Y99E/Y138E) mutant.

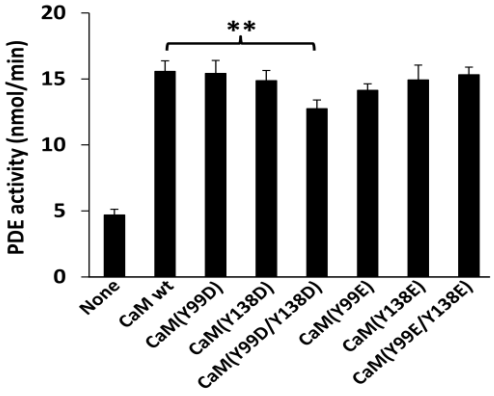

Figure 17: Effect of the different CaM species on the activity of PDE1. The cyclic nucleotide PDE1 activity was assayed in the absence (None) and presence of the indicated CaM species (1.9 µM) in the presence of 100 µM free Ca^{2+} as described in Materials and Methods. The plot presents the average ± SEM of triplicate samples from three separate experiments (** p = 0.02 using the Student's t test).

We also performed the PDE1 assay at increasing concentrations of the CaM mutant species, as compared to wild type CaM, and in agreement with the previous result CaM(Y99D/Y138D) showed no significant differences in the K_{act} (~ 5-10 nM), but a small decrease (~ 20 %) in the V_{max} of the enzyme (Figure 18A). The single CaM(Y99D) and CaM(Y138D) did not presented any differential effect on the PDE1 activation and they behaved as CaM wild type. No significant differences in the K_{act} and the V_{max} of the enzyme were detected when CaM Y/E mutants were tested in the same assay conditions, and all the Y/E mutants behaved as CaM wild type (Figure 18B).

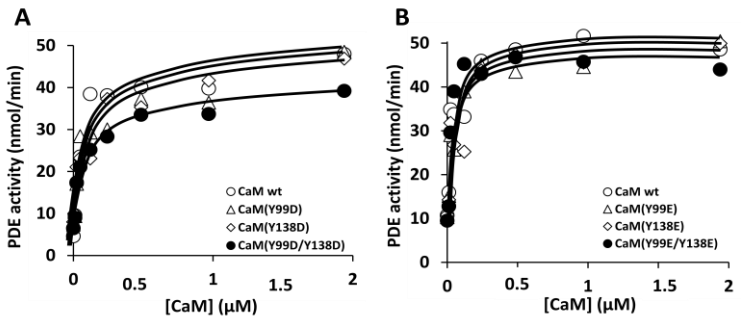

Figure 18: Effect of different concentrations of wild type CaM and the different CaM mutants on the activity of PDE1. The plots present the PDE1 activity assayed as in Figure 17 but using increasing concentrations of CaM Y/D (A) and CaM Y/E (B) mutants.

When the assays were performed at increasing concentrations of free Ca^{2+} no significant changes in the apparent affinity for free Ca^{2+} (~ 5 μM) between wild type and the double Y/D and the double Y/E CaM mutants was noticed (Figure 19). And again a decrease (~ 20-30 %) in the CaM-dependent activity of PDE1 at saturating concentrations of free Ca^{2+} was observed with CaM(Y99D/Y138D), unlike with CaM(Y99E/Y138E), who presented similar activatory effect as compared to the wild type CaM (Figure 19).

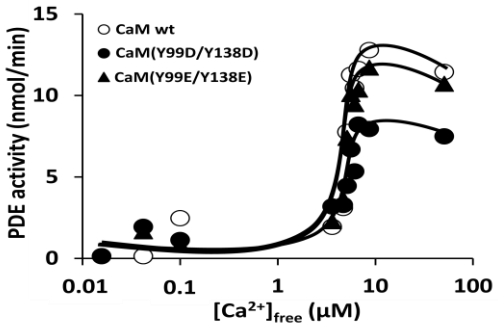

Figure 19: Effect of different Ca^{2+} concentrations on the CaM-dependent PDE1 activity. The plot presents the PDE1 activity assayed as in Figure 17 in the presence of the indicated CaM species (1.9 μM) at increasing concentrations of free Ca^{2+} using an EGTA/Ca^{2+} buffer as described in Materials and Methods.

We also performed PDE1 assays using isothermal titration calorimetry. Serial injections of cAMP were made into the sample cell loaded with PDE1, in the absence or presence of CaM wild type or the CaM(Y99D/Y138D) mutant (3.8 μM). Figure 20A shows heat production over time from serial injection of cAMP until the reaction reaches the plateau and the system was completely saturated. We determined the apparent enthalpy of the reaction ΔH_{app} to be -19.9 ± 0.5 kcal/moles, indicative of no product inhibition in experiments similar to the one shown in Figure 20A, but instead of injecting serial dilution we injected saturating conditions of cAMP. Knowing the enthalpy of the reaction we then performed the experiment in the absence or presence

of wild type CaM and CaM(CaMY99D/Y138D). Figure 20B shows a typical experiment where the presence of wild type CaM greatly enhanced PDE1 activity, while CaM(Y99D/Y138D) had a lesser effect.

We were able to determine from the data presented in Figure 20 the kinetic parameters of the reaction. We determined in a series of similar experiments that the PDE1 activity increased 2.61 ± 0.22 and 1.94 ± 0.09 ($p<0.03$) folds upon addition of wild type CaM and CaM(Y99D/Y138D), respectively.

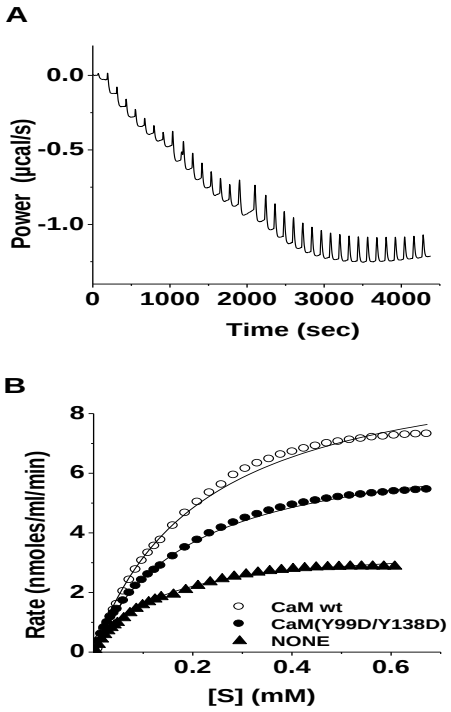

Figure 20: Effect of different CaM species on the kinetics of PDE1 determined by isothermal titration calorimetry. (A) The trace corresponds to a typical experiment showing the rate of heat produced by PDE1 over time after injections of different pulses of cAMP from which the activity of the enzyme can be derived as described in Materials and Methods. This particular trace corresponds to an experiment performed in the absence of CaM using 0.04 units of PDE1. (B) The plot presents the PDE1 activity (normalized for 0.04 units of enzyme) at increasing concentrations of cAMP in the absence (filled triangles) and presence of wild type CaM (open circles) or CaM(Y99D/Y138D) (filled circles) (3.8 µM) in a buffer containing 50 mM imidazol-HCl (pH 7.5), 200 mM NaCl, 5 mM $MgCl_2$, 0.4 mM EGTA, and 0.5 mM $CaCl_2$ using ITC as described in Materials and Methods setting the micro-calorimeter chamber at 37 ºC.

Moreover, the V_{max} was determined to be 7.4 ± 2.8 and 5.5 ± 2.3* ($p<0.05$) nmols/s in the presence of wild type CaM and CaM(Y99D/Y138D), respectively, while the K_m of the enzyme for cAMP was the same (221 ± 27 µM) in the presence of both CaM species (Table 4).

CaM species	V_{max} (exp) (nmols/sec)	Km (fit) (µM)	V_{max} (fit) (nmols/sec)
None	1.8±1.1	170.3 ±94.5	2.4±1.5
CaM wt	5.3±2.1	221.6 ±17.6	7.4±2.8
CaM (Y99D/Y138D)	3.9±1.3	221 ±37.3	5.5±2.3*

Table 4: Kinetic values of PDE1 in the absence and presence of CaM wild type and CaM(Y99D/Y138D).

6.2.2 Effect of the phospho-(Y)-mimetic CaM mutants on eNOS activity

First we tested the comparative action of non-phosphorylated-CaM and phospho-(Y)-CaM on the activation of recombinant eNOS. Figure 21A shows that phospho-(Y)-CaM increased the activity of eNOS more efficiently (~ 30 %) than non-phosphorylated CaM, suggesting that phospho-(Y)-CaM have higher capacity to activate eNOS. When the assay was performed at increasing concentrations of free Ca^{2+} no significant changes in the apparent affinity for free Ca^{2+} (~ 4-6 µM) when compared to non-phosphorylated CaM and phospho-(Y)-CaM was observed (Figure 21B).

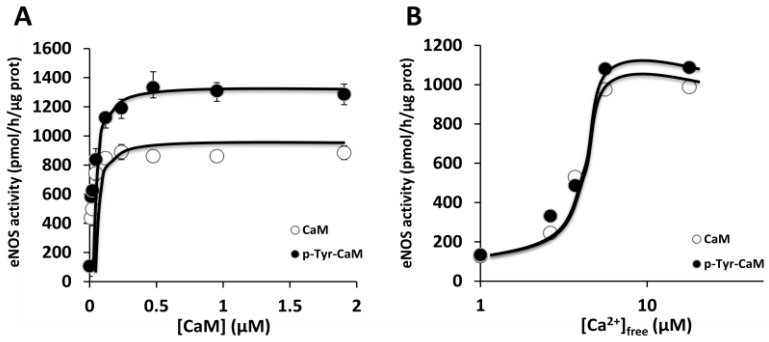

Figure 21: Effect of phospho-(Y)-CaM and non-phosphorylated CaM on the activity of eNOS. (*A*) The plot presents the average ± range activity of eNOS of two independent experiments assayed at increasing concentrations of non-phosphorylated CaM (*open symbols*) or phospho-(Y)-CaM (*filled symbols*) prepared as described in Materials and Methods. (B) The plot presents the activity of eNOS assayed at increasing concentrations of free Ca^{2+} in the presence of non-phosphorylated CaM (*open symbols*) or phospho-(Y)-CaM (*filled symbols*) prepared as described in Materials and Methods.

We tested the comparative action of wild type CaM, CaM(Y99D/Y138D) and CaM(Y99E/Y138E) on the activation of recombinant eNOS. Figure 22A shows that both double Y/D and Y/E CaM mutants strongly activate eNOS in assays performed in the presence of Ca^{2+}, and this activation was slightly more efficient than when wild type CaM was used. When eNOS was assayed at increasing concentrations of the different CaM species (Figure 22B), no significant differences in the K_{act} (~ 0.1 µM) were observed between wild type and the double Y/E CaM mutant. In contrast, a slight increment (~ 30 %) in the V_{max} compared to wild type CaM was noticeable with CaM(Y99E/Y138E). When we tested the effect of the CaM Y/D and Y/E double mutants on eNOS activity at increasing concentrations of free Ca^{2+} no significant changes in the apparent affinity for free Ca^{2+} (~ 4–6 µM) compared to wild type CaM was detected (Figure 22C).

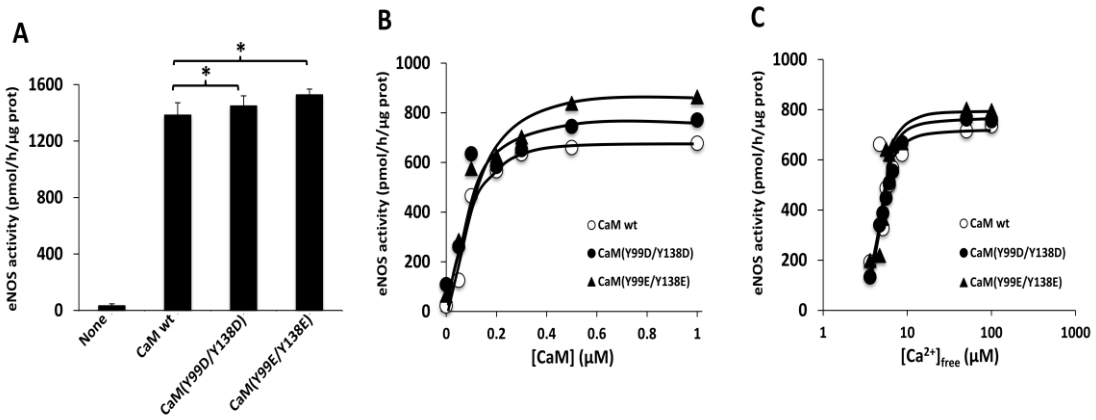

Figure 22: Effect of different CaM wild type and the double Y/D(E) CaM mutants on the activity of eNOS. (*A*) eNOS was assayed in the absence (*None*) and presence of the indicated CaM species (4.7 µM) in the presence of 1 mM free Ca^{2+} as described in Materials and Methods. The plot presents the average ± SEM of triplicate samples from three separate experiments ($p \leq 0.05$ using the Student's t test). (*B*) The plot presents the eNOS activity assayed as in *A* but using increasing concentrations of the indicated CaM species. (*C*) The plot presents the eNOS activity assayed as in *A* in the presence of the indicated CaM species (4.7 µM) at increasing concentrations of free Ca^{2+} using an EGTA/Ca^{2+} buffer as described in Materials and Methods.

6.3 CaM regulates c-Src in Ca^{2+}-dependent and Ca^{2+}-independent manners

The proto-oncogene product c-Src and its related oncoprotein v-Src play important roles in tumor progression and metastasis (Parsons and Parsons, 2004, Wheeler et al., 2009). Notable among the upstream receptors activating c-Src mediating strong metastatic responses are those coupled to G proteins (GPCR), and RTKs, such as EGFR (Parks and Ceresa, 2014) and ErbB2/Her2, as the later promotes the expression and stability of c-Src (Tan et al., 2005).

6.3.1 Apo-CaM and Ca^{2+}/CaM both interact with c-Src

We first performed a typical Ca^{2+}-dependent CaM-affinity chromatography using a detergent-solubilized membrane fraction from A431 tumor cells in order to determine whether Src binds CaM in a Ca^{2+}-dependent manner. Figure 23A shows that actually at least a fraction of the c-Src loaded in the column in the presence of Ca^{2+} can be eluted with a buffer containing EGTA.

As this technique does not show whether part of Src remained bound to CaM-Sepharose in the absence of Ca^{2+}, we performed pull-down experiments using Sepharose with immobilized CaM in the absence and presence of Ca^{2+}. Figure 23B shows that Src binds CaM, both in the presence of Ca^{2+} and its absence, while no significant binding of Src was detected using control CaM-free naked Sepharose beads. Furthermore, we demonstrated that CaM co-immunoprecipitated with Src

solubilized from A431 cells (Figure 23C). Overall, these experiments show most likely a direct interaction between CaM and Src and that this interaction occurs by both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, in agreement with previous findings (Yuan et al., 2011). We also performed CaM overlay and cross-linking experiments with biotinylated CaM to test whether CaM binds to a recombinant or membrane solubilized Src kinase from A431 cells. We were not able to detect, however, any binding neither in the overlay or the cross-linking assays.

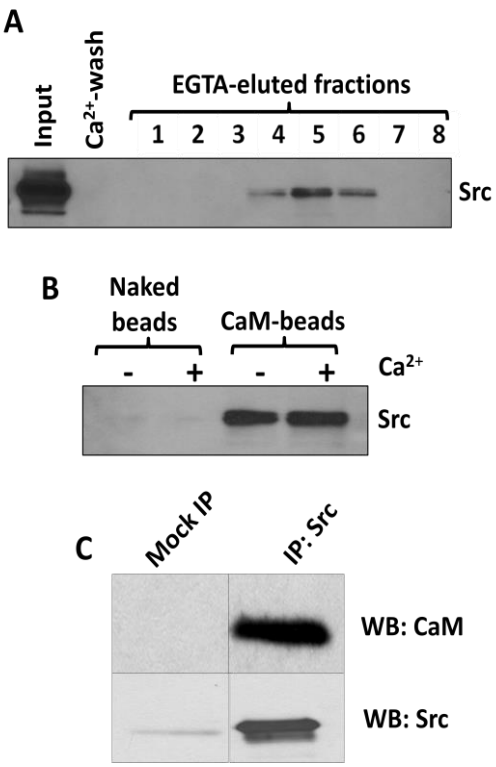

Figure 23: CaM interacts with Src in the absence and presence Ca^{2+}. (*A*) Ca^{2+}-dependent calmodulin-affinity chromatography of Src solubilized from A431 cells was performed as described in Materials and Methods. The input, the last fraction after washing with the Ca^{2+}-buffer (*Ca^{2+}-wash*), and the EGTA-eluted fractions were analyzed by Western blot using an anti-Src antibody. (*B*) Pull-down of Src solubilized from a membrane fraction of A431 cells was performed in the absence and presence of Ca^{2+} using CaM-Sepharose as described in Materials and Methods. Naked Sepharose 4B beads were used as negative binding control. (*C*) Src was solubilized from a membrane fraction of A431 cells (2 mg protein) and immunoprecipitated (IP) using an anti-Src antibody as described in Materials and Methods. The immunocomplex was processed by Western blot using anti-CaM and anti-Src antibodies. Mock IP using rabbit IgG was used as a negative control.

Significant evidence has been obtained on the activatory action of Ca^{2+}/CaM on the tyrosine kinase activity of Src (Fedida-Metula et al., 2012, Yang et al., 2013, Yuan et al., 2011). However, it was generally accepted that this process is always preceded by the generation of a Ca^{2+} signal upon cell activation required for the formation of the Ca^{2+}/CaM complex. Little is known, however, on the possible action of apo-CaM (Ca^{2+} free state) on the activation of c-Src. To solve this question, we tested the effect of CaM on the auto-phosphorylation (activation) of human c-Src in the absence and presence of Ca^{2+} using a full-length recombinant protein. Figures 24A and 24B show that little auto-phosphorylation of c-Src was observed in the absence of CaM and that the presence of this Ca^{2+} sensor strongly enhances c-Src activation in both absence and presence of Ca^{2+}. The activation of recombinant c-Src was stronger in the absence of Ca^{2+} than in its presence. And surprisingly, this effect was also observed

when the basal activity of c-Src was assayed in the absence of CaM. This may suggests a direct inhibitory action of Ca^{2+} on the Src kinase.

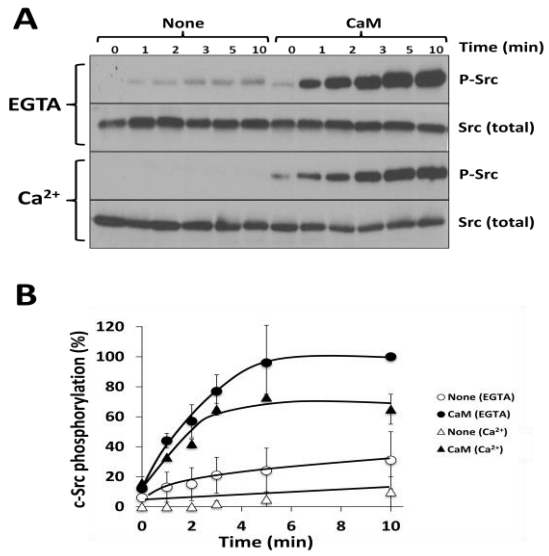

Figure 24: Apo-CaM and Ca^{2+}/CaM both activate recombinant c-Src. (*A*) The auto-phosphorylation assay of recombinant c-Src was performed in the absence and presence of CaM and in the absence and presence of Ca^{2+} for the indicated times as described in Materials and Methods. Duplicate samples were probed with an anti-Src (total) antibody as loading control. (*B*) The plot presents the mean ± range c-Src phosphorylation in the absence (*None*) and presence of CaM, and in the absence (*EGTA*) and presence of Ca^{2+} from two independent experiments similar to the one shown in *A*.

6.3.2 Effect of the phospho-(Y)-mimetic CaM mutants on c-Src activity.

As CaM is known to be phosphorylated by different Src family kinases (Benaim and Villalobo, 2002), we tested the action of phospho-(Y)-mimetic CaM mutants, on the auto-phosphorylation of recombinant c-Src. Figure 25 shows little if any differences in the activatory action of wild type CaM compared with the CaM(Y99D/Y138D) mutant both in the absence and presence of Ca^{2+}. Moreover, both CaM(Y99D/Y138D) and CaM(Y99E/Y138E) had the capacity to activate c-Src more strongly in the absence of Ca^{2+} (presence of EGTA) than in its presence (Figures 25 and 26).

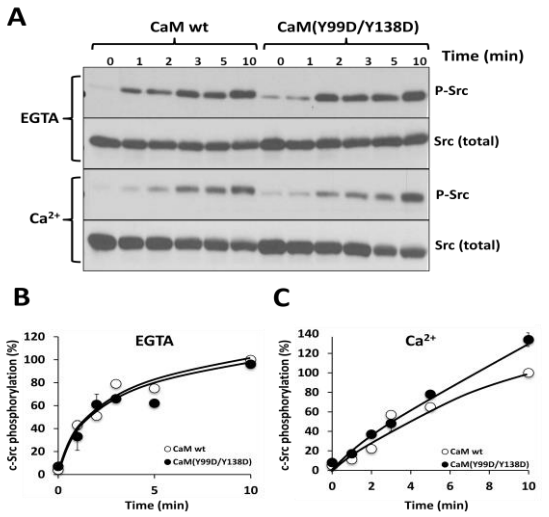

Figure 25: A phospho-(Y)-mimetic CaM mutant activates recombinant c-Src. (A) Auto-phosphorylation assays of recombinant c-Src (0.1 µg) was performed in a medium containing 15 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA and where indicated 1.2 mM $CaCl_2$ (200 µM free Ca^{2+}) in the presence of either wild type CaM or CaM(Y99D/Y138D). The samples were processed for SDS-PAGE and Western blot using anti-phospho-tyrosine and anti-Src antibodies as described in Materials and Methods. (B, C) The plots present the mean ± range (n = 2) c-Src phosphorylation in the presence of wild type CaM or CaM(Y99D/Y138D) in the absence and presence of free Ca^{2+} from experiments similar to the ones shown in A.

These results suggest that phospho-(Y)-CaM may not have a distinct regulatory role than non-phosphorylated CaM on c-Src activation.

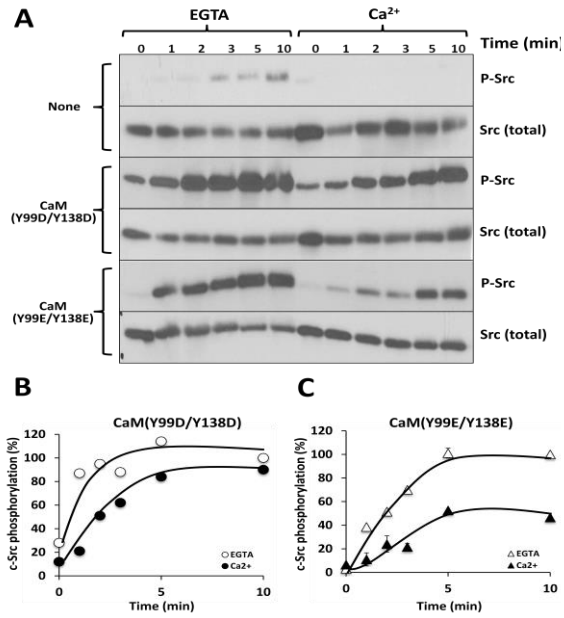

Figure 26: Phospho-(Y)-mimetic CaM mutants activate recombinant c-Src with better efficiency in the absence than in the presence of Ca^{2+}. (A) Auto-phosphorylation assays performed in conditions described in Figure 25 in the absence or presence of either CaM(Y99D/Y138D) or CaM(Y99E/Y138E). The samples were processed for SDS-PAGE and Western blot using anti-phospho-tyrosine and anti-Src antibodies as described in Materials and Methods. (B, C) The plots present the mean ± range c-Src phosphorylation in the presence of CaM(Y99D/Y138D) (n=1) or CaM(Y99E/Y138E) (n=2), and in the absence and presence of free Ca^{2+} from experiments similar to the ones shown in A.

6.3.3 The calmodulin antagonist W-7 inhibits Src activation in living cells.

Ligand-dependent activation of EGFR results in the down-stream activation of Src (Parks and Ceresa, 2014). To determine whether CaM is involved in EGFR-mediated Src activation in EGFR-overexpressing A431 cells, we performed experiments in the presence of the CaM antagonist W-7 and the less potent inhibitor W-12. Figures 27A (*left and center panels*), 27B and 27D show that activation of the EGFR by its ligand EGF also results in Src phosphorylation at Y416 as expected, and that increasing concentrations of W-7 progressively inhibit both phosphorylation processes. In contrast, high concentration (50 µM) of W-12, a chlorine-free analogue of W-7 with far lower affinity for CaM (IC_{50} of 260 µM versus 28 µM, inhibiting Ca^{2+}/CaM-dependent phosphodiesterase) (Hidaka et al., 1981, Hidaka and Tanaka, 1983), has a much lower inhibitory effects on EGFR activation than on Src activation (Figure 27D). It has been previously demonstrated that in living cells the ligand-dependent activation of the EGFR was aided by the Ca^{2+}/CaM complex, and that W-7 or W-13 inhibits this process (Li et al., 2004, 2012, Sengupta et al., 2007). Nevertheless, as the inhibitory action of W-7 on Src phosphorylation was significantly stronger than that observed on ligand-dependent EGFR auto(trans)phosphorylation, this suggests a direct activatory effect of CaM on Src activity. Interestingly, W-7 itself had an activatory effect on the basal Src kinase activity, effect that may be due to the fact that this inhibitor may bind to the phospholipids on the inner leaflet of the plasma membrane and thus change its electrostatic surface potential (Sengupta et al., 2007). The latter event may be

accompanied of the detachment of a region in the Src structure that serves as an autoinhibitory mechanism. Similar effect of W-7/W-13 was also observed in EGFR, as treatment of a variety of cells lines expressing EGFR with this inhibitor led to the activation of the receptor in the absence of ligand (Sengupta et al., 2007).

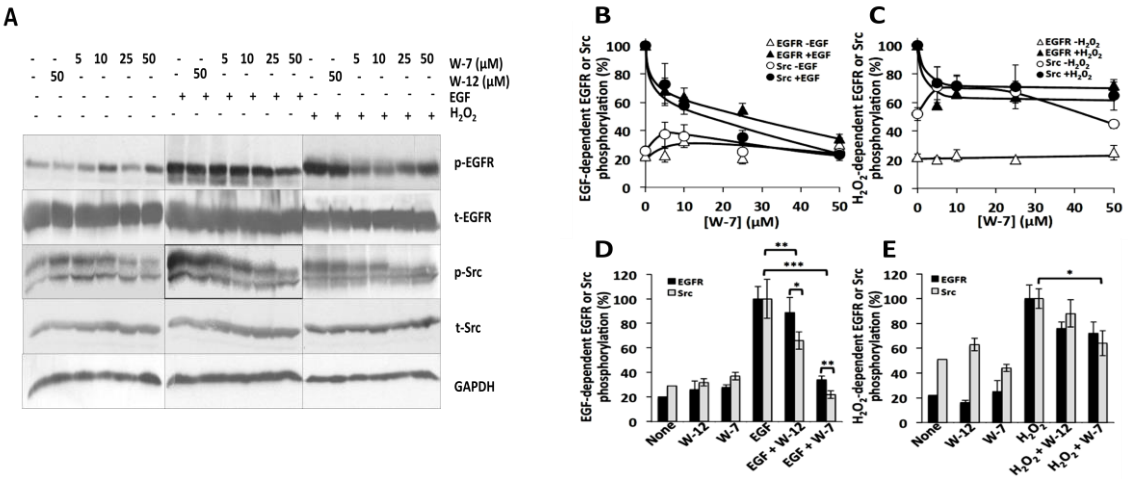

Figure 27: W-7 inhibits EGFR- and H_2O_2-mediated Src activation in A431 cells. (*A*) A431 cells were incubated in the absence and presence of the indicated concentrations of W-12 or W-7 during 15 min. Thereafter, the cells were stimulated either with 10 nM EGF for 2 min or 1 mM H_2O_2 for 15 min. The reaction was arrested and subjected to Western blot analysis as described in Materials and Methods, and probed with anti-phospho-tyrosine (4G10), anti-phospho-Src (Y416), and anti-EGFR (total), anti-Src (total) and anti-GAPDH antibodies as loading controls. (*B, C*) The plots present the mean ± SEM EGF-dependent (n = 6) and H_2O_2-dependent (n = 3) activation of Src at increasing concentrations of W-7 from experiments similar to those shown in *A*. (*D, E*) The plots present the mean ± SEM EGF-dependent (n = 6) and H_2O_2-dependent (n = 3) activation of Src in the absence (*None*) and presence of 50 μM W-12 or 50 μM W-7 from experiments similar to those shown in *A*. Statistically significant differences with $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.0001$ (***) using the Student's t-test are indicated.

The results discussed above do not represent a clear-cut evidence for a direct action of CaM on Src activation in living cells as EGFR is regulated by CaM (San José et al., 1992, Martin-Nieto and Villalobo, 1998, Sengupta et al., 2007, Li et al., 2004, 2012). Therefore, we searched for another way to activate Src without the involvement of EGFR to test the possible inhibitory effect of W-7. Figures 27A (*right panel*), 27C and 27E show that hydrogen peroxide, a known activator of Src (Sato et al., 2001, Basuroy et al., 2010), strongly enhances Src auto-phosphorylation (activation) in A431 cells as expected, and that W-7 indeed significantly inhibits this process in contrast to the lower effect of the low-affinity CaM inhibitor W-12 at identical concentrations (Figure 27E).

We also tested the inhibitory action of W-7 on H_2O_2-dependent activation of Src in a different cell line. Figure 28A show that H_2O_2 enhances the phosphorylation (activation) of Src in SK-BR-3 cells, and that the CaM antagonist W-7 inhibits the H_2O_2-dependent activation of Src while W-12 does not. As W-7 appears to inhibit CaM in its Ca^{2+}-bound form (Okawa et. al, 1998) and may not affect apo-CaM, these results

clearly suggest that Ca^{2+}/CaM regulates Src activity in living cells, although no information can be extracted from these experiments on the potential control that apo-CaM may exert on Src activation.

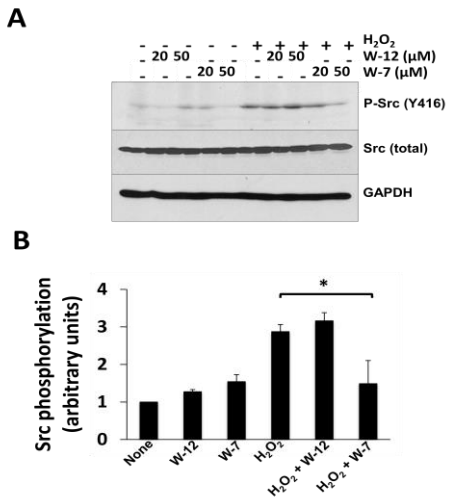

Figure 28: W-7 inhibits H_2O_2-mediated Src activation in SK-BR-3 cells. (*A*) SK-BR-3 cells were incubated in the absence and presence of the indicated concentrations of W-12 or W-7 during 15 min. Thereafter, the cells were stimulated with 1 mM H_2O_2 for 15 min. The reaction was arrested and subjected to Western blot analysis as described in Materials and Methods, and probed with anti-phospho-Src (Y416), and anti-Src (total) and anti-GAPDH antibodies as loading controls. (*B*) The plot presents the mean ± SEM (n = 5) H_2O_2-dependent activation of Src in the absence (*None*) and presence of either 50 μM W-12 or 50 μM W-7 from experiments similar to the one shown in *A*. Statistically significant differences with $p < 0.05$ (*) using the Student's t-test are indicated.

We tried to test the effect of CaM on the activity of Src family kinases espressed in ET1-55/EGFR cells, where the expression of CaM is down-modulated by tetracycline (Panina et al., 2012, Li et al., 2012), using as control cell line DT40/EGFR cells. After the antibiotic treatment for 48 hours we detected with a surprise an increase in the phosphorylation at Tyr416 of some unidentified member of the Src-family, but this was not due to the decrease of the expression of CaM, because this increased phosphorylation was also detected in the control cell line, suggesting that stress induced by tetracycline, but not the drop of CaM expression level, was the reason for the activation of the Src family kinase(s) (Supplementary Figure 2S).

6.4 Effect of CaM and phospho-(Y)-CaM on EGF-dependent EGFR activation

Overexpression, and/or truncated, site-mutated or segment-deleted hyperactive forms of EGFR are present in many solid tumors in human. The aberrant receptors significantly contribute to the oncogenic process (Kim and Muller, 1999, Roskoski, 2014). Thus, targeting overexpressed and/or aberrant hyperactive EGFR could be a valid therapeutic approach against cancer (Roskoski, 2014). In addition to anti-EGFR antibodies targeting the extracellular region of the receptor, small chemicals targeting the tyrosine kinase domain of EGFR have been developed. Another target site of interest could be the CaM-BD located at the cytosolic JM region of the receptor (Villalobo et al., 2013).

6.4.1 Effect of CaM down-regulation in CaM-KO cells on EGF-dependent EGFR activation.

As CaM is implicated in EGF-dependent EGFR activation we used ET1-55/EGFR conditional CaM-KO cells stably transfected with the human EGFR (Panina et al., 2012, Li et al., 2012). Upon addition of tetracycline the expression level of CaM drops after 42-72 h of exposure of the antibiotic and we could barely detect the presence of CaM by immunoblot (Figure 29A). The activation of the EGFR was significantly reduced by tetracycline addition in a time-dependent manner and the decrease of EGFR auto-phosphorylation follows the time-dependent down-regulation of CaM, showing that CaM is essential for EGF-dependent activation of the EGFR (Figures 29A and 29) as previously described by us (Li et al., 2012). Since CaM is vital for the cells, we used trypan blue exclusion test to determine the cell viability at the different times of tetracycline treatment, and at 72 h only 10 % drop in cell viability was observed compared to non-treated cells (Figure 29C).

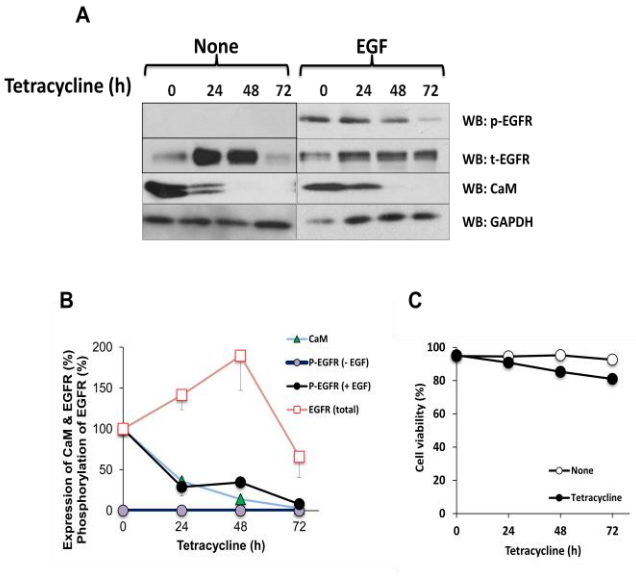

Figure 29: CaM down-regulation in conditional CaM-KO cells decreases EGF-dependent EGFR activation. (A) ET1-55/EGFR cells were incubated with 1 µg/ml tetracycline and stimulated with 10 nM EGF for 5 min for the indicated times. The expression of EGFR (t-EGFR), phospho-EGFR (p-EGFR) and CaM was determined using an anti-EGFR, anti-phospho-tyrosine (4G10) and anti-CaM antibodies. GADPH is also shown as a loading control. (B) The plot presents the mean ± SEM (n=5 for total EGFR, n=3 for phospho-EGFR, and n=3 for total CaM) expression of total EGFR (*red squares*), non-stimulated EGFR (*purple circles*), EGF-stimulated EGFR (*black circles*) and total CaM (*green triangles*) in the presence of tetracycline for the indicated times. (C) The plot represents the mean ± SEM (n=3) of the cell viability measured by trypan blue exclusion test in the absence and presence of tetracycline for the indicated times.

6.4.2 Effect of the phospho-(Y)-mimetic CaM mutants on EGFR activation.

Then we wanted to test whether the double Y/D and Y/E CaM mutants have a differential regulatory effect on the EGF-dependent activation of the EGFR as compared to CaM wild type. Figure 30 shows the effect of the double Y/E and Y/D mutants on the EGF-dependent activation of EGFR solubilized from a membrane fraction of A431 cells *in vitro*. The results show that the receptor in the absence of its ligand has very low tyrosine kinase activity, and that upon addition of EGF leads to a strong activation of the EGFR. The effect of CaM wild type is in agreement with what has been already demonstrated by us, where the presence of Ca^{2+}/CaM has an inhibitory effect on the EGF-dependent activation of EGFR *in vitro* (San José et al., 1992). Interestingly, CaM(Y99E/Y138E) and CaM(Y99D/Y138D) mutants had a differential effect on the activation of the receptor in the presence of Ca^{2+}. The glutamic acid double mutant had similar, if not stronger, inhibitory effect compared to the one exerted by wild type CaM. On the other hand, the aspartic acid mutant did not exhibit the same extend of inhibition on the EGF-dependent activation of EGFR in the presence of Ca^{2+}, compared to both wild type CaM and the double Y/E CaM mutant. In the absence and presence of Ca^{2+} Y/D CaM mutant had a strong activatory effect on the EGF-dependent phosphorylation of EGFR as compared to wild type CaM (Figure 30B).

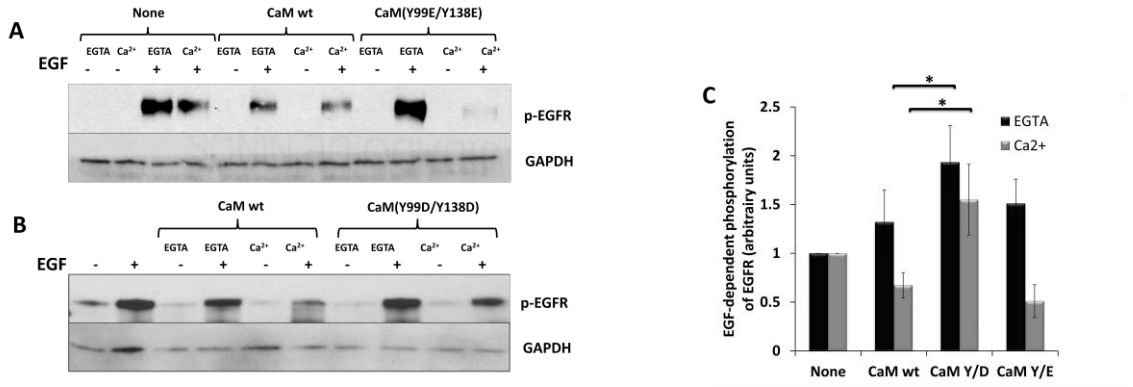

Figure 30: Effect of different CaM species on the EGF-dependent phosphorylation of the EGFR *in vitro*. (A, B) EGFR solubilized from membrane fraction from serum-starved A431 cells was incubated in the absence or presence of different recombinant CaM mutants (1 µg), in the absence of Ca^{2+} (presence of EGTA) or presence of Ca^{2+}, and in the absence or presence of 10 nM EGF as indicated. The samples were resolved in SDS-PAGE and processed by immunoblot using an anti-phospho-tyrosine antibody to detect phosphorylated EGFR (p-EGFR). GAPDH is shown as a loading control. (C) The plot presents the mean ± SEM (n = 6) for wild type CaM and CaM (Y99D/Y138D), and (n=3) for CaM(Y99E/Y138E) of the EGF-dependent phosphorylation of EGFR in the absence (presence of EGTA) or in the presence of Ca^{2+} and the different CaM species as indicated.

6.5 *O*-GlcNAcylation in tumor cells

O-GlcNAcylation is the addition of β-D-N-acetylglucosamine moiety(ies) to serine or threonine of nuclear, cytoplasmic and mitochondrial proteins (Hart, 2014). Since 1984 when *O*-GlcNAcylation was found for the first time (Torres and Hart, 1984) significant amount of data revealing the function exerted by this non-typical glycosylation has been accumulated, and its involvement in diseases such as cancer has crystallized in recent years (Hart, 2014, de Queiroz et al., 2014, Jozwiak et al., 2014). It was found that EGFR type III in *Drosophila* is subjected to *O*-GlcNAcylation (Sprung et al., 2005) and it has been proposed based on *in silico* studies that Th654 and Ser1046/1047 in the EGFR are *O*-GlcNAcylated (Kaleem et al., 2009). The putative *O*-GlcNAcylation sites proposed for the EGFR are in fact directly or indirectly regulated by CaM, as Thr654 is located in the CaM-BD of the receptor and Ser1046/1047 are phosphorylated by CaMK-II. Thus we tried to peer whether the EGFR is in fact subjected to this PTM in tumor cells and whether CaM could have any role in this process.

6.5.1 EGFR *O*-GlcNAcylation in A431 and A549 cells

We first tested for the presence of *O*-GlcNAcylated proteins in whole cell lysates of A431 tumor cells by immunoblot using two distinct anti-*O*-GlcNAc specific antibodies (CTD110.6 and RL2) (Figure 31A). The presence of multiple strongly labeled bands of *O*-GlcNAcylated proteins in a range of > 250 kDa to < 50 kDa were detected presenting a similar distribution pattern when both antibodies were used. When cells were treated with Thiamet G, a highly specific inhibitor of *O*-GlcNAc hydrolase (OGA) to prevent *O*-GlcNAc deglycosylation (Goldberg et al., 2011), a significant two-fold increase in protein *O*-GlcNAcylation level was detected as expected (Figures 31B, 31C and 31D). Moreover, we also tested BADGP, a general *O*-glycosylation inhibitor not specific for *O*-GlcNAcylation but that also inhibits this process (Filhoulaud et al., 2009, Pantaleon et al., 2010, Onodera et al., 2014, Xu et al., 2014), and a small but significant decrease in protein *O*-GlcNAcylation was detected (Figures 31B and 31C).

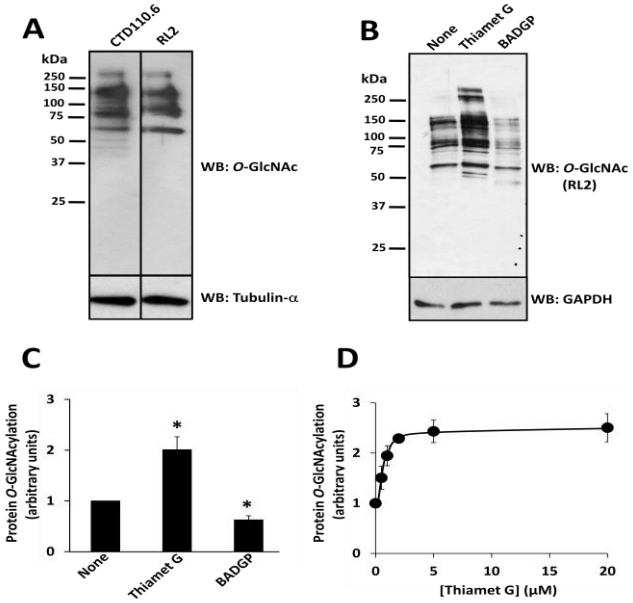

Figure 31: Effects of OGT and OGA inhibitors on protein *O*-GlcNAcylation. (A) A cell extract from A431 cells was processed by SDS-PAGE and Western blots (WB) and probed with the anti-*O*-GlcNAc antibodies CTD110.6 and RL2 as indicated. (B) A431 cells were incubated overnight in the absence (None) and presence of 20 μM Thiamet G or 2 mM BADGP as indicated and whole cell extracts probed with an anti-*O*-GlcNAc antibody (RL2). Anti-GAPDH antibody was used as loading control. (C) The plot presents the mean ± SEM (n = 4) of total protein *O*-GlcNAcylation. (*) $p < 0.05$ as determined by the Student's t test. (D) The plot presents the mean ± SEM (n = 3) protein *O*-GlcNAcylation using different concentrations of Thiamet G.

6.5.2 EGFR *O*-GlcNAcylation detected by immunoblot

To determine whether the EGFR from A431 tumor cells undergoes *O*-GlcNAcylation we treated the immunoprecipitated receptor in the absence and presence of PNGase F to remove *N*-glycans, and tested for *O*-GlcNAcylation using the same two distinct anti-*O*-GlcNAc antibodies mentioned above (Figures 32A and 32B). The results show that in both instances, not only the 170 kDa native EGFR, but most significantly and in greater extent the ~150 kDa *N*-deglycosylated receptor, yielded positive signals. To ascertain the specificity of the CTD110.6 anti-*O*-GlcNAc antibody, we competed its reactivity using an excess (20 mM) of free GlcNAc in the incubation medium (Figure 32C). Furthermore, we performed the experiment in reverse: immunoprecipitating first *O*-GlcNAcylated proteins from an A431 cell lysate and detecting the presence of EGFR in the immunoprecipitate (Figure 32D). When the cells were previously treated with the OGA inhibitor Thiamet G, the EGFR signal was slightly increased, and a decrease was detected when cells were treated with the *O*-glycosylation inhibitor BADGP, which also inhibits OGT (Figure 32D).

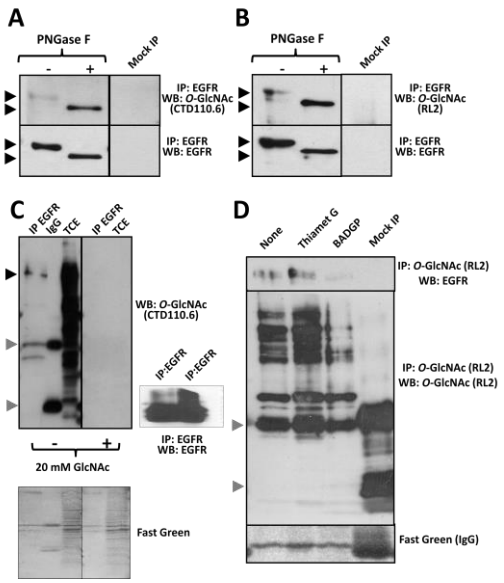

Figure 32: Detection of *O*-GlcNAcylation signal in immunoprecipitated EGFR by immunoblot. (*A, B*) The EGFR was immunoprecipitated (IP) from A431 cells using an anti-EGFR antibody and the immunocomplex was incubated in the absence and presence of PNGase F. The samples were blotted with the anti-*O*-GlcNAc antibodies CTD110.6 (*A*) and RL2 *(B*). The membranes were reprobed with an anti-EGFR antibody as loading controls. Mock IP were performed using a non-relevant IgG as negative controls. Arrowheads point the native and *N*-deglycosylated EGFR. (*C*) Immunoprecipitated (IP) EGFR, a non-relevant IgG fraction and an A431 cell extract were immunoblotted using an anti-*O*-GlcNAc antibody (CTD110.6) in the absence and presence 20 mM GlcNAc. (*D*) A431 cells were incubated overnight in the absence (*None*) and presence of 20 µM Thiamet G or 2 mM BADGP. The samples were immunoprecipitated (IP) using an anti-*O*-GlcNAc antibody (RL2) and the immunocomplex subjected to Western blots (WB) and probed with anti-EGFR and anti-*O*-GlcNAc antibodies. Mock IP was performed using a non-relevant IgG as negative control. The light chains of IgG were staining with Fast Green as loading control.

In agreement with the previous result, we also demonstrated that when A431 cells were treated with the OGA inhibitor Thiamet G the *O*-GlcNAcylation level of the immunoprecipitated native 170 kDa EGFR also increased, although little if any increment was detected in the ~150 kDa *N*-glycans-free EGFR band (Figures 33A and 33B).

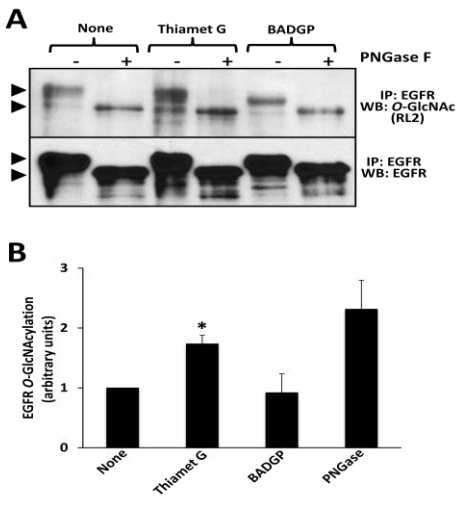

Figure 33: Effects of OGA and OGT inhibitors on the EGFR *O*-GlcNAcylation. (A) A431 cells were incubated overnight in the absence (None) and presence of 20 µM Thiamet G or 2 mM BADGP. The samples were incubated in absence or presence of PNGase F and processed as described in Materials and Methods and probed with an anti-*O*-GlcNAc antibody (RL2). The membrane was reprobed with an anti-EGFR antibody as loading control. Arrowheads point the native and *N*-deglycosylated EGFR. (B) The plot presents the mean ± SEM (n = 3) EGFR *O*-GlcNAcylation from a set of experiments similar to the one shown in A. (*) $p < 0.05$ as determined by the Student's t test.

We then tested whether EGFR activation with EGF has an effect on the *O*-GlcNAcylation of the receptor, and on the *O*-GlcNAcylation of total proteins in A431 cells. Figures 34A and 34B show that when EGFR is activated by EGF there is a small, but not significant decrease in the *O*-GlcNAcylation of the receptor, this may be partially due to the internalization and degradation of the receptor induced by its ligand (Wiley et al., 1991, Lamaze and Schmid, 1995, Oksvold et al., 2003, Henriksen et al., 2013) (see Figures 35C and 35B). Figure 34C shows that EGF-stimulated A431 cells have the same pattern of *O*-GlcNAcylated proteins compared to the non-treated ones, suggesting that EGFR signaling does not modulate this PTM in other proteins.

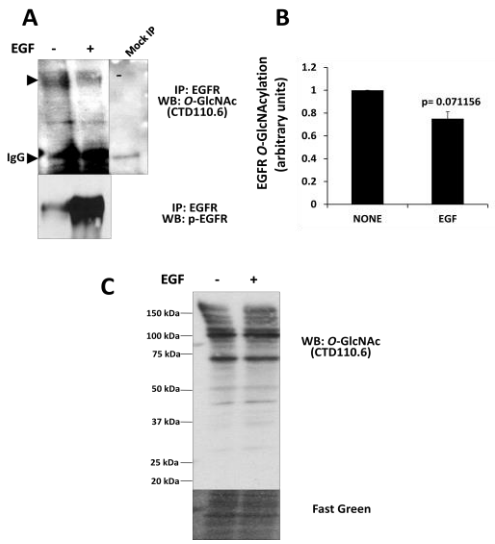

Figure 34: The effect of EGF-dependent activation on the *O*-GlcNAcylation of the EGFR. (A) Serum-starved A431 cells were incubated in the absence and presence of 10 nM EGF during 30 min. EGFR was immunoprecipitated and immunoblotted (WB) with the anti-*O*-GlcNAc antibody CTD110.6. (B) The plot presents the mean ± SEM (n = 3) EGFR *O*-GlcNAcylation. The p-value as determined by the Student's t test is also shown. (C) Cell extracts of serum-starved A431 cells were incubated in the absence and presence of 10 nM EGF for 30 min. The samples were then processed as described in Materials and Methods and probed with the anti-*O*-GlcNAc antibody.

6.5.3 EGFR *O*-GlcNAcylation detected by metabolic labeling with azido-GlcNAc

We next tried to detect *O*-GlcNAcylation of the EGFR by metabolically labeling cellular glycans from A431 cells with azido-GlcNAc. Figure 35A shows that after immunoprecipitation both the native 170 kDa EGFR and its ~150 kDa *N*-glycans-free form, obtained after PNGase F treatment, yielded a positive signal following biotinylation and overlay by streptavidin-HRP. The extent of the signal was lower in EGF-treated cells but this most likely represents degradation of the receptor after ligand-induced internalization, as this process has been well documented (Kirisits et al., 2007, Henriksen et al., 2013) and we further show this degradation in Figures 35B, 35C. We also treated A431 cells with tunicamycin to obtain EGFR devoid of *N*-glycans by a different method. Figure 34D shows that the native 170 kDa receptor and its ~150 kDa *N*-deglycosylated form both were metabolically labeled with azido-GlcNAc, the latter even after further treating the immunoprecipitated EGFR with PNGase F.

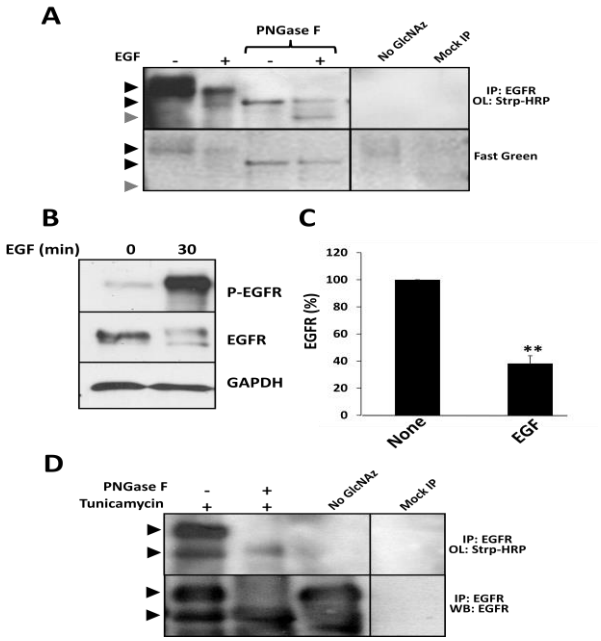

Figure 35: Detection of *O*-GlcNAcylation signal in EGFR by azido-GlcNAc labeling. (A) Serum-starved A431 cells were labeled with GlcNAz and incubated in the absence and presence of 10 nM EGF during 30 min. EGFR was then immunoprecipitated, biotinylated and N-deglycosylated as described in Materials and Methods. The detection of the biotin-GlcNAc complex was done by overlaid with streptavidin-HRP as described in Materials and Methods. Immunoprecipitated EGFR from cells non-treated with GlcNAz (No GlcNAz) and a mock IP are shown as negative controls. Fast Green protein staining is shown as loading control. (B) Serum-starved A431 cells were treated in the absence (-) and presence (+) of 10 nM EGFR for 30 min. The reaction was arrested with 10 % (w/v) TCA, and the samples were processed for SDS-PAGE and immunoblot using an anti-phospho-tyrosine antibody to detect phosphorylated EGFR (P-EGFR) and an anti-EGFR antibody to detect total EGFR. GAPDH is shown as a loading control. (C) The plot presents the mean ± SEM (n = 3) of the densitometry of the EGFR/GAPDH signal ratio. (**) $p < 0.01$ as determined by the Student's t test. (D) A431 cells were treated overnight with 1 µg/ml tunicamycin, the EGFR was immunoprecipitated (IP) and where indicated treated with PNGase F. The samples were processed as in A. Duplicate samples were probed with an anti-EGFR antibody. Immunoprecipitated EGFR from cells non-treated with GlcNAz (No GlcNAz) and a mock IP are shown as negative controls. Arrowheads point the native and *N*-deglycosylated EGFR.

We tested different human tumor cell lines, with different levels of EGFR expression, and a mouse fibroblast stably transfected and overexpressing the human EGFR for *O*-GlcNAcylation of the receptor. Figure 36 shows a positive signal in human carcinoma epidermoid A431 cells, which were used as positive control. Also, a positive signal was detected in human lung carcinoma A549 cells. The double band observed in A549 cells may represent the presence of an unrelated *O*-GlcNAcylated protein associated to the EGFR and/or another ErbB receptor forming heterodimers with the EGFR. However, no *O*-GlcNAcylation of the EGFR was detected in human cervix adenocarcinoma HeLa cells or in the recombinant human EGFR expressed in mouse EGFR-T17 fibroblasts.

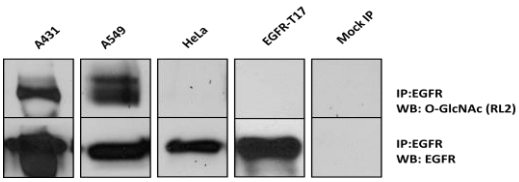

Figure 36: *O*-GlcNAcylation of EGFR in different cell lines. A431, A549, HeLa and EGFR-T17 cells were incubated overnight in the presence of the OGA inhibitor 20 µM Thiamet G. The EGFR was immunoprecipitated (IP) using an anti-EGFR antibody and the immunocomplexes were processed by SDS-PAGE and immunoblot (WB) using anti-*O*-GlcNac (RL2) and anti-EGFR antibodies as described in Materials and Methods. A mock IP was performed using a non-relevant IgG as a negative control.

6.5.4 *In vitro* O-GlcNAcylation of EGFR

We first show that EGFR and OGT can be co-immunoprecipitated (Figure 37A), suggesting that the two proteins interact in living cells. Our results on the *in vitro* enzymatic assays show a significant increment in the *O*-GlcNAcylation level of the EGFR as detected by Western blot using an anti-*O*-GlcNAc antibody (RL2) (Figures 37B and 37C). Given the fact that the immunoprecipitated EGFR was already partially *O*-GlcNAcylated before starting the reaction catalyzed by OGT, this increment only reached ~1.8 ± 0.2 fold. This, however, strongly suggests that the EGFR undergoes this PTM.

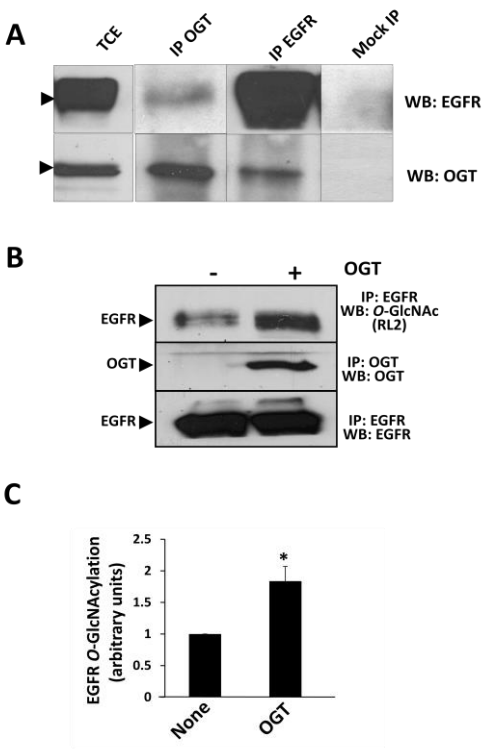

Figure 37: Enhanced *O*-GlcNAcylation of EGFR upon *in vitro* reaction catalyzed by OGT. (A) OGT and EGFR were immunoprecipitated from A431 cells and the samples were processed by immunoblot (WB) using anti-OGT and anti-EGFR antibodies to test for co-immunoprecipitation of both proteins. Mock IP were performed using a non-relevant IgG as negative controls. (B) OGT and EGFR were independently immunoprecipitated from A431 cells. Thereafter, the immunoprecipitated EGFR was incubated in the absence and presence of immunoprecipitated OGT and the *O*-GlcNAcylation was performed upon addition of UDP-GlcNAc as described in Materials and Methods. The samples were immunoblotted as indicated. OGT and EGFR were detected by WB using anti-OGT and anti-EGFR antibodies, respectively. (C) The plot presents the mean ± SEM EGFR O-GlcNAcylation in the absence (None) and presence of OGT from 3 independent experiments similar to the one shown in B measuring the densitometry of the O-GlcNAcylated EGFR band corrected by loading as determined by protein staining with Fast Green. (*) $p < 0.05$ as determined by the Student's t test.

6.5.5 Effect of OGA and OGT inhibitors on EGFR activation

We tested OGA and OGT inhibitors on the EGF-dependent activation of the EGFR in A431 tumor cells. Figure 38A shows that the presence of Thiamet G and BADGP, did not significantly change the time-dependent activation profile of the EGFR. The maximum EGFR phosphorylation was around 2-10 minutes of EGF stimulation followed by a subsequent decrease of the receptor auto-phosphorylation due to dephosphorylation (Figure 38B).

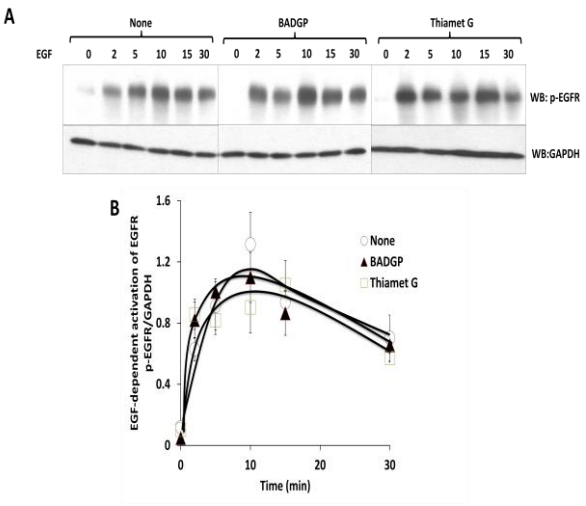

Figure 38: Effect of OGA and OGT inhibitors on the EGF-dependent activation of EGFR. (*A*) A431 cells were incubated in the absence (*None*) and presence of 20 μM Thiamet G or 2 mM BADGP overnight. Thereafter, the cells were stimulated with 10 nM EGF for the indicated times. The reaction was arrested and subjected to Western blot analysis as described in Materials and Methods, and probed with anti-phospho-tyrosine antibody (p-EGFR), and anti-GAPDH antibody as a loading control. (*B*) The plot present the mean ± SEM (n=3) EGF-dependent activation of EGFR for the indicated times from experiments similar to those shown in *A*.

6.5.6 Possible role of CaM in protein *O*-GlcNAcylation

According to Kaleem *et al.* (2009) CaM could have a central role into the proposed interplay between *O*-GlcNAcylation and phosphorylation of the EGFR. We tried to study the possible role of CaM on OGT functionality in order to address this issue and clarify whether this Ca^{2+} modulator has anything to do with the *O*-GlcNAc modification. Figure 39 shows an *in silico* study proposing that OGT could be a CaM-BP. Using a CaM database *in silico* tool freely available at: http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html we were able to find a sequence located in the C-terminal of the transferase that has great potential of being a CaM-BD (Figure 39A). Since we know that CaM-BD regions of a given protein are usually conserved among different species we tested whether the putative CaM-BD of OGT is also conserved, and the result of the alignment of this region is shown in Figure 39B. It is clear that this region is quite conserve as there is only one amino acid substitution, a valine at position 993 changed to isoleucine, both hydrophobic amino acids (Figure 39B). On the other hand, the putative CaM-binding site was identified as a canonical 1-5-10 class motif (Figure 39C) (see also Table 1).

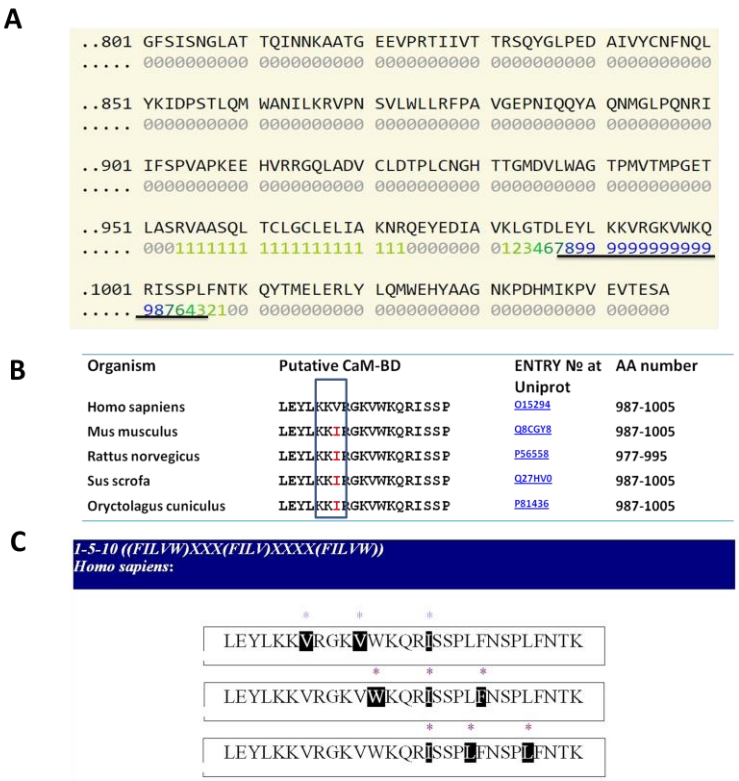

Figure 39: Putative CaM-binding domain in human ncOGT. (A) Presents the result from the *in silico* searching tool showing the amino acid sequence in the human ncOGT that have the highest potential to present CaM binding capacity (from 0 to 9). (B) Alignment of the selected region in different species. (C) The scheme represents the position of the conserved amino acids critical for CaM binding and several segments fitting to a CaM-binding domain of type 1-5-10.

We performed Ca^{2+}-dependent CaM-affinity chromatography using recombinant ncOGT in order to determine whether this enzyme binds CaM in a Ca^{2+}-dependent manner. Figure 40A shows that part of the OGT loaded in the column in the presence of Ca^{2+} can be eluted with a buffer containing EGTA. Furthermore, we demonstrated that CaM co-immunoprecipitated with OGT from A431 cells (Figure 40B). Overall, these experiments show possible direct interaction between CaM and OGT and that this interaction occurs by a Ca^{2+}-dependent mechanism.

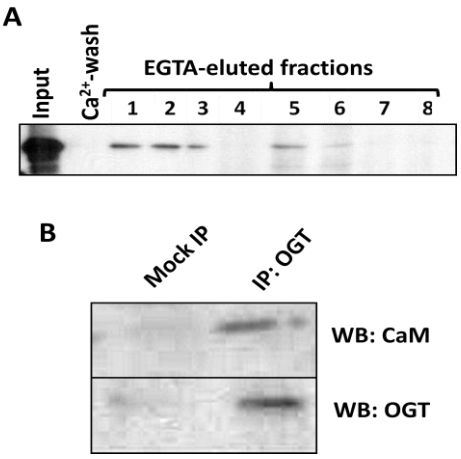

Figure 40: Calmodulin interacts with OGT. (*A*) Ca^{2+}-dependent CaM-affinity chromatography of recombinant ncOGT performed as described in Materials and Methods. The input, the last fraction after washing with the Ca^{2+}-buffer (*Ca^{2+}-wash*), and the EGTA-eluted fractions were analyzed by Western blot using an anti-OGT antibody. (*B*) OGT was immunoprecipitated (IP) from A431 cells using an anti-OGT antibody as described in Materials and Methods. The immunocomplex was processed by Western blot using anti-CaM and anti-OGT antibodies. A mock IP was used as a negative control.

We finally tested the effect of CaM inhibition and CaM down-regulation on the *O*-GlcNAcylation pattern of A431 cells and ET1-55/EGFR cells, respectively. Figures 41A and 41B show that neither the inhibition of CaM with W-7 in A431 cells nor the down-regulation of CaM in the conditional CaM-KO chicken lymphoma cell line has any effect on the general pattern of protein *O*-GlcNAcylation, suggesting that CaM may not regulate the enzymatic activity of OGT.

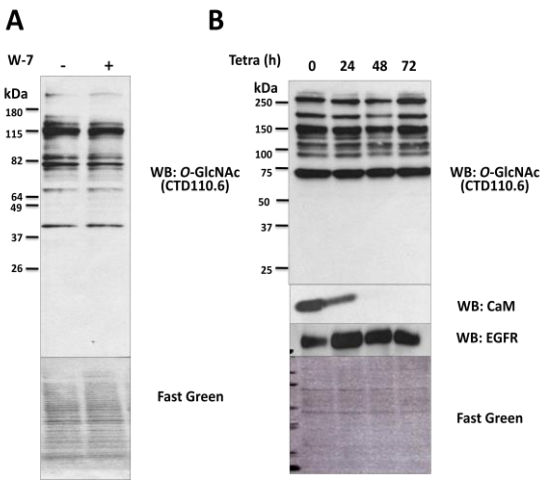

Figure 41: Effect of inhibition or down-regulation of CaM on the O-GlcNAcylation pattern. (A) A431 cells were treated in the absence or presence of 15 µM W-7 for 30 min. Total cell extracts samples were processed by SDS-PAGE and Western blot as described in Material and Methods, and the membrane was probed with anti-*O*-GlcNAc (CTD110.6) antibody. Protein stained with Fast Green is shown as a loading control. (B) ET1-55/EGFR cells were treated with tetracycline (1 µg/ml) for the indicated times. Total cell extracts were processed by SDS-PAGE and Western Blot as described in Material and Methods and the membranes probed with anti-*O*-GlcNAc (CTD110.6), anti-CaM, anti-EGFR antibodies. Protein stained with Fast Green is shown as a loading control.

7. DISCUSSION

CaM is a modulator in eukaryotic cells transducing the Ca^{2+} signal generated upon all activation by a variety of effectors, and regulates many cellular processes including: cell proliferation, apoptosis and autophagy, all of them playing a fundamental role in tumorigenesis and tumor progression (Chin and Means, 2000, Jurado et al., 1999, Berchtold and Villalobo, 2014). Understanding the different mechanisms by which CaM exerts its functions, regulating hundreds of proteins including EGFR and c-Src, that also have been identified as important elements of signaling pathways that control the invasive and metastatic capacities of various tumors (Kim and Muller, 1999, Roskoski, 2014, Tan et al.,2005) is of a highest interest. In this Thesis we focus on studing the effect of CaM on the functionality on systems important in tumorogenesis, particularly the EGFR and c-Src.

A broad range of Ser/Thr- and Tyr-protein kinases have been shown to phosphorylate CaM *in vitro* and in living cells and the different phospho-CaM species have different regulatory role on the activity of many target proteins as compared to non-phosphorylated CaM (Benaim and Villalobo, 2002). This suggests that this PTM, in addition to Ca^{2+} binding, could modulate the activity of CaM. The non-receptor tyrosine kinase Src (Fukami et al., 1986), the insulin receptor (Graves et al., 1986), and the EGFR (Benguría et al., 1994), are kinases implicated in the phosphorylation of CaM at both Y99 and Y138. The dynamic nature of this PTM and the distinct phosphorylated forms of CaM at a given time acting on specific target proteins could be difficult to observe in living cells due to the short half-life of phospho-(Y)-CaM. In this Thesis we show an alternative to explore these events by generation and characterization of recombinant phospho-(Y)-mimetic CaM mutants, where one or two of the two only Tyr present in CaM were substituted by glutamic acid (E) or aspartic acid (D), as this is a common approach used to mimic tyrosine phosphorylation (Potter et al., 2005, Lie et al., 2012, Fisslthaler et al., 2008). We generated and extensively characterized the phospho-mimetic potential of these CaM mutants.

7.1 The phospho-(Y)-mimetic CaM mutants binds Ca^{2+}

Our data showed, that in contrast to the homogeneous electrophoretic migration (~ 18 kDa) of the single or double Y/D and Y/E CaM mutants in the absence of Ca^{2+} (presence of EGTA), and the near absence of Ca^{2+}-induced electrophoretic mobility shift of the single or double CaM mutants where Y138 was substituted by acidic residues (D or E) described in this Thesis, the non-phosphorylatable Y/F CaM mutants previously described presented a different behavior (Salas et al., 2005). For instance, the CaM(Y138F) and CaM(Y99F/Y138F) mutants presented in the absence of Ca^{2+}

higher electrophoretic mobility than wild type CaM and CaM(Y99F), and even higher Ca^{2+}-induced mobility shift (Salas et al., 2005). This suggests that the substitution of Y138 by an acidic or a hydrophobic residue exerts distinct effects on the conformation of CaM and most likely have a great impact on the conformation of the protein. Our results are in agreement with previous findings in which a differential behavior was observed when Y138 or Y99 were substituted by diverse amino acids, suggesting that mutation of Y138 disrupts the structural coupling between the N and C globular halves of CaM potentially mimicking the collapse that CaM undergoes when binding to target proteins (Sun et al., 1999, Sun et al., 2001). The central position of Y138 in the Ca^{2+}-induced conformational change of CaM is also evidenced by the modification of the chiroptical properties of this residue, here revealed to be the culprit for the well-known changes in the near-UV CD spectrum of CaM. Of note, the spectrum of Ca^{2+}-bound rat CaM is very similar to that of *Drosophila melanogaster* CaM (Maune et al., 1992), which only contains the Y138 residue but not that at position 99, further substantiating this conclusion.

The far-UV CD spectra of the different phospho-mimetic CaM species as compared to the wild type in the absence or presence of Ca^{2+} suggest that the substitution of one or the two Tyr residues does not disrupt Ca^{2+} binding. Ca^{2+} binding induced similar conformational changes in the molecules of the whole panel of mutants. Nevertheless, the ratio of molar ellipticity at 208 and 222 nm, a sensitive criterion of possible alterations in interactions between neighboring helices (Sun et al., 2001, Fasman, 1996), was slightly higher for the mutants as compared to the wild type, pointing to slight differences in the helix packing of the mutants. Of note, the mutant-specific deepening of the signals in the presence of Ca^{2+} may be due to a differential increase in α-helical content and/or reorientation of existing α-helices (Wang et al., 2011, Protasevich et al., 1997). Interestingly, all CaM mutant species presented a slight blue-shift of the 208 nm minimum in the absence of Ca^{2+} as compared to wild type CaM. We could not differentiate if this blue-shift was due to a solvent-effect or because of a mechanism including the involvement of aromatic-side chains. However, the fact that the blue-shift was more noticeable in the spectra of the two double CaM mutants suggests that most likely this shift of 2-3 nm is due to a reorganization of the molecule, because of the lack of the two tyrosines.

On the other hand, the near-UV spectra of wild type CaM showed similar features as to what has been published so far (Sun et al., 2001, Wolff et al., 1977, Martin and Bayley, 1986). The spectrum consisted of two well-defined negative bands at 262 and 269 nm, attributed to the six phenylalanine residues presented in CaM, and a region

around 280 nm arising from the two Tyr residues. All the latter picks reacted to Ca^{2+} binding and presented the typical spectra deepening. Unlike the mutants, which presented the expected change in the region of 280 nm, the spectra in this region became in the case of Y99 mutants even positive, and in the case of Y138 mutants the ellipticity became almost zero. Our results clearly show that Ca^{2+}-induced alterations observed for wild type CaM in this region are mostly due to changes in the environment of Y138. Interestingly, this conclusion fully explains the small effect of Ca^{2+} on the near-UV CD spectrum of the separate N-terminal half (residues 1-77) of CaM reported by others (Martin and Bayley, 1986). They considered their result as a surprise, because of the equal distribution of the phenylalanine residues in the C- and N-terminal halves of the protein. Actually, the spectra with/without Ca^{2+} of the Y138 and double CaM mutants are very similar to those published for the N-terminal half by the same group (Martin and Bayley, 1986).

In agreement with the previous reports showing that Ca^{2+} binding induces a notable increase in the thermal stability of wild type CaM (Wang et al., 2011, Brzeska et al., 1983), our results clearly showed that the full panel of mutants preserve this spectacular quality. All the CaM species showed quasisigmoidal decrease in ellipticity in the absence of Ca^{2+}, clearly indicating the occurrence of at least one intermediate state, as previously described for wild type CaM (Sun et al., 2001, Wang et al., 2011). In the case of the CaM Y138 mutants, the temperature-dependent unfolding was clearly biphasic, showing a notable deviation of sigmoidicity at temperatures below 50 ºC. The behavior of the Y138 mutant is remarkably similar to that reported for a CaM Y138Q mutant (Sun et al., 2001). Although, fitting the data to a two- or three-state model did not yield acceptable quality, it is clear from the denaturation profiles that a major thermal unfolding for the different CaM species occurred at ~ 55 ºC, and all of them were completely unfolded above 70 ºC. In addition, lower temperature transitions and/or discontinuities, also reported for wild type CaM (Brzeska et al., 1983, Martin and Bayley, 1986, Kilhoffer et al., 1981, Gangola and Pant, 1983), were observed in the lower temperature region (20-40 ºC), both in the absence and presence of Ca^{2+}. Most importantly, the binding of Ca^{2+} had a profound impact on the stability of wild type CaM and all of the mutants, they exhibited remarkably thermal stability in the presence of Ca^{2+}, showing that ion binding has a similar stabilizing effect on the wild type and the mutants. This conclusion, together with the far-UV CD spectrum is also supported by the fact that the full panel of CaM mutants required Ca^{2+} to stimulate the activities of PDE1 and eNOS, as it was the case for wild type CaM. Therefore, the absence of a

significant Ca^{2+}-induced electrophoretic mobility shift in the Y138D and Y138E CaM mutants is not a valid criterion to propose absence of Ca^{2+}-binding capability.

It has been previously reported that terbium ion binds to the four Ca^{2+}-binding pockets of CaM and that it can be excited indirectly by excitation of the tyrosine residues at 280 nm. The amount of energy transferred from the excited tyrosine to the terbium ion depends on how close the tyrosine and Tb^{3+} are. Thus, this indirect excitation of Tb^{3+} could be monitored at 545 nm (Wallace et al., 1982). This fact allowed us to study the binding of Tb^{3+} to the different CaM mutant species. The near loss of fluorescence emission by Tb^{3+} upon tyrosine excitation in the single mutant lacking Y99, but not the single mutant lacking Y138, suggests that Tb^{3+} is closer to tyrosine 99 located at the III Ca^{2+}-binding site of CaM, than to tyrosine 138 located at its IV Ca^{2+}-binding site. This is in full agreement with the crystallographic structure of CaM, where the carbonyl group of Y99 participates in the coordination of Ca^{2+}, while Y138 has a rather outward projecting conformation unable to coordinate Ca^{2+} (Strynadka and James, 1989).

In agreement with the distinct spatial projection and accessibility to kinases of the two tyrosine-residues of CaM, our c-Src phosphorylation experiments show that Y99 was phosphorylated with lower efficiency than Y138 by this kinase. The inward projection of Y99 within the III Ca^{2+}-binding pocket as compared to a more outward projection of Y138 within the IV Ca^{2+}-binding pocket (Strynadka and James, 1989) may explain these results. Although both tyrosine residues in CaM are phosphorylated by both c-Src and EGFR, as demonstrated using Y99F and Y138F CaM mutants (Salas et al., 2005), the substitution of the tyrosine residue at position 138 by a non-phosphorylatable amino acid somehow disturbs in part the accessibility of these kinases to Y99. This is remarkable, as it was previously suggested that phosphorylation of Y99 by several tyrosine kinases was more efficient than the phosphorylation of Y138 (Benaim and Villalobo, 2002). Thus, the phosphorylation of single-tyrosine CaM mutants may not reflect the comparative efficiency of phosphorylation of both tyrosine residues of CaM in living cells. This may be related to the fact that substituting Y138 by other amino acids disrupts the structural coupling between the N- and C-globular domains of CaM (Sun et al., 2001), as previously mentioned.

7.2 All phospho-(Y)-mimetic CaM mutants are biologically active

Our results also show that the single and double Y/D and Y/E CaM mutants all retain biological activity, as they were able to activate distinct CaM-binding systems

including PDE1, eNOS, EGFR, and c-Src. Moreover, we have shown a differential action of wild type CaM and some of the phospho-(Y)-mimetic CaM mutants under study on the activity of PDE1, eNOS and EGFR but not on c-Src.

The double mutant CaM(Y99D/Y138D), but not CaM(Y99E/Y138E), exerts a lower activatory effect on the V_{max} of the CaM-dependent cyclic nucleotide PDE1 isolated from bovine brain, as compared to wild type CaM. However, the aspartic acid mutant does not significantly change the apparent affinity for Ca^{2+} or the activation constant (K_{act}) of CaM for the enzyme. Of note is that other studies on the effect of phospho-(Y)-CaM versus non-phosphorylated CaM on the cyclic nucleotide phosphodiesterase of different origin yielded divergent results. Thus, both an increase (Williams et al., 1994) and a decrease (Corti et al., 1999) in the K_{act}, without affecting the V_{max}, has been reported for phospho-(Y)-CaM phosphorylated by the InsR and a phospho-(Y99)-CaM species, respectively, on the PDE1 from bovine brain in comparison to non-phosphorylated CaM. In contrast, InsR-phosphorylated CaM presented similar K_{act} and V_{max} than with non-phosphorylated CaM when assayed on the PDE1 from rat hepatocytes, although the phosphorylated form increased the IC_{50} of CaM antagonists inhibiting the PDE1 activity (Saville and Houslay, 1994). In radical contrast, phospho-(Y)-CaM, phosphorylated by the EGFR, inhibited by 90 % the activity of the PDE1 from bovine heart (Palomo-Jiménez et al., 1999). These conflicting data may be due to the fact that at least eleven families of distinct cyclic nucleotide PDEs, including different isoforms of the CaM-dependent PDE1 with distinct affinities for cAMP and/or cGMP and diverse regulatory properties may coexist and/or being differentially expressed at distinct ratios in various tissues and/or cell types in diverse organisms (Francis et al., 2011). Alternatively, distinct phospho-Y99/phospho-Y138 ratios in the sample of phospho-(Y)-CaM used may account for the observed discrepancies. Nevertheless, it is likely that the negative charges of the extra aspartic acids present in CaM(Y99D/Y138D) may exert a similar action that phospho-CaM in some PDE1 isoform(s). Furthermore, the absence of effect of the single Y/D mutants suggests that the phosphorylation of both tyrosine residues may be necessary for the lower activatory action of phospho-(Y)-CaM on PDE1. The absence of significant effect of the CaM(Y99E/Y138E) mutant also suggests that the length of the side chain of glutamic acid, longer than the one of aspartic acid (3.39 Å and 2.56 Å, respectively, from the carboxylic to the C-α) (Allen, 2002), may be unfitted to interact with the PDE1 site where phospho-(Y)-CaM exerts its regulatory action on the enzyme.

We show that the mutant CaM(Y99D/Y138D), and most prominently CaM(Y99E/Y138E), slightly increase (~ 20-30 %) the V_{max} of eNOS as compared to

wild type CaM. Other studies, however, have shown that the single mutant CaM(Y99E) slightly increase (16 %) the activity of recombinant iNOS, has no significant effect on nNOS, and significantly inhibited (40 %) the activity of eNOS (Piazza et al., 2012). This suggests that the different NOS isoforms may be regulated differently and that the phosphorylation of either one or the two tyrosine residues of CaM may exert significant differential effects on its activity. Nevertheless, our results are qualitatively, but not quantitatively, in agreement with the stimulatory action that phospho-(Y99)-CaM exerts on the V_{max} of nNOS, where a 3.4-fold higher V_{max} than with non-phosphorylated CaM was reported (Corti et al., 1999). These authors also reported a two-fold higher K_{act} of phospho-(Y99)-CaM with respect to non-phosphorylated CaM, and a 4-fold lower K_d for a peptide corresponding to the CaM-binding domain of nNOS (Corti et al., 1999). The different NOS isoforms used in these studies could explain the discrepancy with the lower phospho-mimetic capacity of CaM(Y99D/Y138D) and CaM(Y99E/Y138E) when compared to tyrosine-phosphorylated CaM observed by us.

We also tested the effect of CaM(Y99E/Y138E) on the eNOS activity determined by ITC technique using increasing concentration of L-arginine. However, we encountered huge difficulties with eNOS that made the result obtained uncertain. First of all, the calculated enthalpy of the reaction was very low (-1.4 ± 0.1 kcal/mol) and only a minute quantity of the heat produced during the reaction was detected. Secondly, the critical step of base-line stabilization was prolonged and took about ~1-2 hours for the base-line to stabilize, time that most likely had a negative impact on the enzyme activity. Moreover, during this stabilization period the enzyme was incubated in a complex medium containing a variety of cofactors; notably NADPH, as electron donor, and FAD/FMN that are essential cofactors and also electron acceptors, short-circuiting the electron flow due to the diaphorase activity of eNOS before L-arginine was added (Grozdanovic and Gossrau, 1995).

Our results with the phospho-mimetic CaM(Y99D/Y138D) and CaM(Y99E/Y138E) mutants suggest that phospho-(Y)-CaM may not have a differential action on c-Src activation as compared to non-phosphorylated CaM. Intriguingly, is has been shown in keratinocytes that phospho-(Y138)-CaM does not co-immunoprecipitate with Src, while non-phosphorylated CaM does (Wu et al., 2015). In contrast, our results using CaM(Y99D/Y138D) and CaM(Y99E/Y138E) suggest that CaM phosphorylated at both tyrosines may be able to interact with Src because increases its auto-phoshorylation, in contrast to what it was reported with CaM phosphorylated at Y138 (Wu et al., 2015). No information, however, is available on the possible action of CaM phosphorylated at Y99 on Src activity. This underscores, nevertheless, the potential importance of distinct

phosphorylated tyrosine residues and/or variable phosphorylation stoichiometry in the regulation of CaM-binding proteins, as it becomes apparent in other systems (Benaim and Villalobo, 2002).

It has been shown previously that the cytosolic JM region of the EGFR is essential for its activation (Hubbard, 2009, Red Brewer et al., 2009, Jura et al., 2009). In the generally accepted theory the JM domain as it contains many positively charged amino acids, serves as an auto-inhibitory mechanism by attaching this part of the protein to the negatively charged inner leaflet of the plasma membrane (McLaughlin et al., 2005). Our group has discovered that within the cytosolic juxtamembrane domain of the EGFR lays a region rich in basic amino acids that in fact are forming a CaM-BD (Martin-Nieto and Villalobo, 1998). Previous reports have demonstrated that when CaM binds to the CaM-BD of the EGFR *in vitro* it inhibits its tyrosine kinase activity in a Ca^{2+}-dependent manner as it mimics the negative charged acidic phospholipids in the plasma membrane (San José et al., 1992). In contrast to this, CaM has an activating role in the EGF-dependent activation of the EGFR in living cells (Li et al., 2004, 2012, Sengupta et al., 2007), as it helps to detach the JM from the plasma membrane (McLaughlin et al., 2005, Sato et al., 2006, Sengupta et al., 2007)

Our *in vitro* assays confirmed previous results showing that wild type CaM inhibits the EGF-dependent tyrosine activity of the EGFR in a detergent-solubilized preparation in a Ca^{2+}-dependent manner (San José et al., 1992). When no Ca^{2+} was present in the sample, CaM cannot bind to the receptor to inactivate it, thus we observed higher ligand-dependent activation of the receptor. Interestingly, as in the case of PDE1 and eNOS, here we also observed a differential role of the CaM mutants. CaM(Y99E/Y138E) behaved exactly as the wild type CaM inhibiting the EGF-dependent activation of the EGFR in the presence of Ca^{2+}, while CaM(Y99D/Y138D) exhibited far lower potential inhibiting the auto-phosphorylation of EGFR *in vitro*. The latter results, together with some yet unpublished results generated in our lab show that phospho-(Y)-CaM free of non-phosphorylated CaM has the ability to activate the EGFR instead of inhibiting it *in vitro*, similar to the one exerted by the CaM(Y99D/Y138D) mutant (Stateva et al., 2015, in preparation). All taken together comes to show that phospho-(Y)-CaM may have even higher potential than non-phosphorylated CaM in activating the receptor in living cells.

It is intriguing that CaM(Y99D/Y138D), but not CaM(Y99E/Y138E), exerts a differential action on PDE1 and EGFR, as compared to wild type CaM; while in the case of eNOS both CaM(Y99D/Y138D) and CaM(Y99E/Y138E) exert similar effect,

albeit the latter with better efficiency. This could be related to the different length of the side chain of glutamic acid and aspartic acid as mentioned above. Thus, although the actual reason for these distinct effects is unknown, we could speculate that each enzyme or tyrosine kinase used in this study may have pockets or clefts of distinct depths where the negative charge of the carboxyl group of the acidic amino acid must adapt in a proper conformation in order to exert its differential action, and that this pocket/cleft may be deeper in PDE1 than in eNOS. Although the phospho-(Y)-mimetic CaM mutants described in this work exert small differential effects with respect to wild type CaM on the two CaM-binding enzymes tested, the observed effects are qualitative consistent with those exerted by phospho-(Y)-CaM. Therefore, these mutants might be useful tools to study the differential regulation exerted by phospho-(Y)-CaM in a wide variety of CaM-binding proteins with and without enzymatic activity in living cells. This could be achieved by the stable expression of these phospho-(Y)-mimetic CaM mutants in a conditional CaM knockout DT40 cell line recently describe (Panina et al., 2012), after the down-regulation of endogenous CaM by tetracycline, where the action of distinct Ca^{2+}-null and other CaM mutants on the viability and proliferation capacity of these cells was tested (Panina et al., 2012).

7.3 CaM interacts with and activates c-Src

In this work we also show that c-Src not only binds CaM in the absence and presence of Ca^{2+}, as others have previously demonstrated (Yuan et al., 2011, Fedida-Metula et al., 2012, Pérez et al., 2013), but that the Ca^{2+}/CaM complex, and more efficiently apo-CaM, both activate c-Src (Stateva et al., 2015 in press). Although the activation of Src by different signals appears to require Ca^{2+}, as demonstrated by the attenuation of Src activation in cells loaded with the Ca^{2+}-chelator BAPTA-AM (Bobe et al., 2003, Samak et al., 2011), the Ca^{2+}-independent action of CaM on Src activation advises for a partial revision of previous findings on the necessary involvement of an executor Ca^{2+} signal preceding the binding of CaM to Src (Fedida-Metula et al., 2012, Yang et al., 2013, Bobe et al., 2003, Samak et al., 2011, Pérez et al., 2013). The drastic reduction of the cytosolic concentration of free Ca^{2+} imposed in BAPTA-loaded cells may also affect other Ca^{2+}/CaM-dependent systems upstream of Src, as for example the EGFR (Martín-Nieto and Villalobo, 1998, Li et al., 2004, 2012), and not exclusively Src activation. Therefore, a transient Ca^{2+} rise may not be an obligatory step to attain activation of Src since apo-CaM exerts this role even more effectively than Ca^{2+}/CaM. One possibility, however, could be that oscillations in the cytosolic concentration of free Ca^{2+} indeed could act as a rate modulator of the activity of the Src/CaM complex, where CaM could be already tethered to Src, as it occurs in other

CaM-binding proteins such as distinct Ca^{2+}-channels (Saucerman and Bers, 2012). Thus, the kinase activity in the Src/CaM complex could decrease when the concentration of Ca^{2+} rises, by occupying the Ca^{2+}-binding sites of tethered CaM, and conversely increases when the concentration of Ca^{2+} falls and the cation is released from CaM. These events may not necessarily be a simple two-steps cycle (Ca^{2+}-free and Ca^{2+}-bound), as CaM has two high-affinity and two low-affinity Ca^{2+}-binding sites (Chin and Means, 2000). Thus, this process may occur as a multi-stage cycle where a variable number of Ca^{2+}-binding sites are free or occupied at a given time, what may result in a variable rate of Src activity and a more fine-tuned regulation of the kinase.

An alternative possibility is that distinct Ca^{2+}-dependent and Ca^{2+}-independent CaM-binding sites could exist in Src. Yuan and collaborators (Yuan et al., 2011) proposed as the CaM-binding site of human Src the sequence ^{199}KHYKIRKLDSGGF^{213}, located in the terminal part of the SH2 domain. However, mutation of this site substituting the sequence KHYKIRKLDS by alanine residues only partially prevented CaM binding (Yuan et al., 2011). This suggests the existence of additional CaM-binding sites in Src. We identified *in silico* in human Src two additional potential CaM-binding sites, that we denote atypical IQ-like motifs, corresponding to the sequences: ^{146}**IQ**AEEWYFG**K**^{155}, located in the proximal region of the SH2 domain, and ^{311}**LQ**EAQVM**KK**L**R**H^{322}, located in the proximal region of the tyrosine kinase domain (Figure 42). Of note, is to mention that the last sequence located at the proximal region of the kinase has a great potential to be a canonical CaM-BD as well (Figure 42). These sites may contribute to the binding of apo-CaM, as many IQ- and related IQ-like motifs are known to be receptor sites for Ca^{2+}-free CaM in different target proteins (Jurado et al., 1999, Rhoads and Friedberg, 1997).

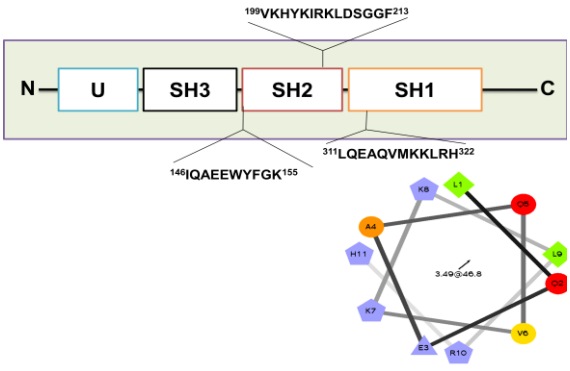

Figure 42: Putative CaM-binding domains in c-Src. The overall structure of Src family kinases is shown, as well as the two putative CaM-binding domains including the potential IQ-like motif. The alpha helical wheel projection of the ^{311}**LQ**EAQVM**KK**L**R**H^{322} sequence is also shown.

The non-receptor tyrosine kinase Src is subjected to a complex regulatory mechanisms mediated by phosphorylation events which control its activation status (Okada and Nakagawa, 1989, Cooper and Howell, 1993, Parsons and Parsons, 2004,

Irtegun et al., 2013). The stabilization of its activation loop induced by auto-phosphorylation of Y416 maintains the kinase in an open conformation, allowing substrate binding and hence subsequent signal transmission by the resulting phosphorylated substrates (Cooper and Howell, 1993). On the other hand, the specific phosphorylation of its C-terminal tail at Y527 by Csk or Chk, maintains Src in a closed conformation repressing its activity (Okada and Nakagawa, 1989).

The identification of Src as a CaM-binding protein, and the proposed location of the CaM-binding site(s) at the SH2 and/or tyrosine kinase domains, suggests that CaM may mediate its action maintaining Src in its open active conformation. We also observed that Ca^{2+} *per se* (absence of CaM) has a direct inhibitory action on Src auto-phosphorylation. This is unlikely to be mediated by an exogenous Ca^{2+}-dependent system, as for example PKC, because we used a purified preparation of recombinant Src and the assay was devoid of cofactors required for PKC activation. Although speculative, an interesting possibility is to search for potential EF-hand Ca^{2+}-binding pocket(s) in Src.

Another point of interest is the fact that the two partners in the well-known bidirectional activatory trans-phosphorylation between EGFR and Src, where Src phosphorylates the EGFR (Anderson et al., 1990, Lombardo et al., 1995, Sato et al., 1995, Biscardi et al., 1998, Gao et al., 2001), and the EGFR phosphorylates Src (Parks and Ceresa, 2014), are regulated by CaM, albeit in different manners. The regulation of EGFR by CaM is a Ca^{2+}-dependent process (Martín-Nieto and Villalobo, 1998, Li et al., 2004, 2012), while the regulation of Src by CaM appears to be a two-faced process, Ca^{2+}-dependent and Ca^{2+}-independent. This opens the door to explore in future work how physiological oscillations in the cytosolic concentration of free Ca^{2+}, upon cell stimulation by mitogenic and/or other factors, modifies this mechanism by affecting in different manner both interconnected partners, the EGFR and Src, and hence the proliferative response.

7.4 EGFR is subjected to *O*-GlcNAcylation in tumor cells

Overall our results show for the first time that human EGFR could be subjected to *O*-GlcNAcylation, as it is the case for the insulin receptor (Yang et al., 2008). We detected EGFR *O*-GlcNAcylation in two distinct human tumor cell lines, A431 and A549 cells. A431 cells, derived from a tumor of epidermic origin, and A549 cells derived from a lung epithelial tumor. In contrast, no signal was detected in the EGFR from HeLa cells that are derived from a virus-induced cervical adenocarcinoma. This demonstrates that EGFR *O*-GlcNAcylation may not occur in all tumor cells, as this

process could depend of its tissular and/or organ origin. Moreover, the absence of EGFR *O*-GlcNAcylation signal in EGFR-T17 mouse fibroblasts suggests that this post-translational modification may not be working in a non-physiological setting where a recombinant human EGFR is artificially expressed in cells of other species.

O-GlcNAcylation normally is expected to take place in the cytosolic region of the EGFR. Nevertheless, it is noticeable that when the immunoprecipitated EGFR was treated with PNGase F the immunoreactivity of the ~150 kDa *N*-deglycosylated receptor to the two anti-*O*-GlcNAc antibodies used increased as compared with the native 170 kDa EGFR. This suggests that removal of *N*-glycans from the extracellular region of the EGFR could facilitate the exposure of occluded GlcNAc moieties linked to complex *O*-glycans, not related with *O*-GlcNAcylation, events that could be non-specifically recognized by the two anti-*O*-GlcNAc antibodies used in this study. In fact, the presence of *O*-glycans in the extracellular region of the EGFR has been described (Wu et al., 2011). Other possibility could be that the removal of sialic acids distributed in the *N*-glycans by the PNGase F enzyme is the reason for both antibodies to have an increased reactivity with the *N*-deglycosylated receptor, as it has been already shown that desialation leads to a better binding of certain antibodies (Bolmstedt et al., 1992, Nguyen et al., 2006). Alternatively, the increased immunoreactivity of the anti-*O*-GlcNAc antibodies after the removal of the *N*-glycans by PNGase F, could be also related to the extracellular *O*-GlcNAcylation of the receptor, modification driven by EOGT (Sakaidani et al., 2011, Sakaidani et al., 2012, Ogawa et al., 2014). We were able to find in the extracellular region of the EGFR three sites (^{215}**C**AQQC**SG**R**C**^{223}, ^{236}**C**AA**G**C**TG**PRE^{245} and ^{506}**C**HALC**S**PE**GC**^{515}) that contain key amino acids (cysteine, glycine and serine/threonine) that are also present in the EGF-like domain consensus sequence **C**XX**G**X**S**/**TG**XX**C** recognized by EOGT (Alfaro et al., 2012), particularly the second one. These sites are in the S1 and S2 cysteine-rich regions of the receptor, and the cysteine residues located in the proposed sequences are involved in the formation of disulfide bridges (Abe et al., 1998). Moreover, the strict specificity of some of the anti-*O*-GlcNAc antibodies has been questioned, as for example the CTD110.6 antibody that also recognizes terminal β-GlcNac in complex *N*-glycans (Tashima and Stanley, 2014).

PNGase F is extensively used to remove *N*-glycans from glycoproteins (Maley et al., 1989), but this endoglycosidase is unable to work if a α1-3 bond links fucose to the innermost GlcNAc attached to asparagine (Tretter et al., 1991). Although α1-3-linked fucose to the innermost GlcNAc residues attached to asparagine is common in plant

and insect glycoproteins, it appears to be absent in mammalian cells, where α1-6-linked fucosylation of core GlcNAc catalyzed by fucosyl transferase 8 (FUT8) appears to be the norm (Ma et al., 2006). Nevertheless, to bypass the possible existence of an unrecognized aberrant α1-3-linked fucosylation of the innermost GlcNAc attached to asparagine in tumor cells we used tunicamycin treatment. Our results with tunicamycin also supports absence of α1-3-linked fucosylation of core *O*-GlcNAc in the EGFR from A431 cells, as GlcNAz labeling was clearly detected in the ~150 kDa *N*-deglycosylated EGFR.

The apparent lower level of *O*-GlcNAcylation on the EGFR when the receptor was exposed to EGF stimulation could be explained by the EGF-driven internalization and degradation of the receptor, as this is one of the major consequences of the ligand-dependent activation of the EGFR (Kirisits et al., 2007, Henriksen et al., 2013). In fact, we could not detect any difference in the pattern of the *O*-GlcNacylation in the whole cell protein extract of A431 cells stimulated with EGF. It seems that EGF-induced activation of the EGFR of these cells does not involve changes in the *O*-GlcNAcylation status of other proteins. The lack of effect of EGF-activation of A431 cells on protein *O*-GlcNAcylation supports the idea that EGFR signaling is not modulating the *O*-GlcNAc machinery. Moreover, we expected that EGFR may be able to phosphorylate OGT as proposed by others (Kaleem et al., 2009). However, the *in vitro* phosphorylation assay that we performed gave negative results and most likely OGT is not a substrate for EGFR (Supplementary figure 3S).

Besides the fact that lectins have limited binding affinity and specificity they have been extensively used in studying protein glycosylation (Yang and Hancock, 2005, Etxebarria et al., 2012, Rosenfeld et al., 2007, Fry et al., 2012). WGA is a lectin recognizing GlcNAc moieties located at any glycan structure (De Hoff et al., 2009, Gallagher et al., 1985), we tried to address the question on whether WGA may be able to bind to PNGase F-deglycosylated EGFR (Supplementary figure 4SA). The lectin was able to bind to the *N*-deglycosylated receptor, suggesting that GlcNAc moiety(ies) recognized by the WGA could be located at Ser/Thr residues (Supplementary figure 4SA). Moreover, in order to find out whether EGFR has more complex *O*-glycan structures in its extracellular part as suggested previously, that may be recognized by the anti-*O*-GlcNAc antibodies we have used in our study (CTD110.6 and RL2) or with WGA and thus giving false positive for EGFR *O*-GlcNAcylation, we probed the immunoprecipitated receptor with PNA, a lectin that has preferences in binding to Ser/Thr attached mucin type *O*-glycans (Tajima et al., 2005) (Supplementary figure

4SB). These glycans were found to be present in EGFR expressed in a hepatocellular carcinoma cell (HCC) line (Wu et al., 2011). In fact, it has been shown that by knocking-down the enzyme responsible for the transfer of this mucin type *O*-glycans on target proteins, GALNT2, the malignancy of HCC cells was significantly reduced through mechanism involving EGFR. However, the fact that PNA was not able to bind to the receptor immunoprecipitated from A431 cells (Supplementary figure 4S), strongly suggests that this mucin type *O*-glycosylation is not taking place in these cells. Therefore, we can exclude the possibility of cross-reaction of the two antibodies or WGA with any mucin type *O*-glycans of the receptor expressed in A431 tumor cells.

The suggestion that the EGFR could be subjected to *O*-GlcNAcylation at Thr654 and Ser1046/Ser1047 based on *in silico* modeling as proposed before (Kaleem et al., 2009) is pending experimental confirmation, as many predicting software programs are prone to both false-positive and false-negative predictions (Jochmann et al., 2014). We attempted some experiments using mass spectrometry (MS) to detect EGFR *O*-GlcNAcylation. However, the lack of peptides corresponding to the cytosolic juxtamembrane region of the EGFR, where Thr654 is located, prevents us attaining meaningful results. Nevertheless, we accumulated significant amount of data suggesting that the receptor is subjected to this PTM (Stateva et al., 2015 submitted). We believe that the identification in human EGFR of *O*-linked GlcNAc moiety/ies and the specific Ser and/or Thr residue(s) where it may be attached using a suitable MS method or another technology could be, if positive, the ultimate proof of EGFR *O*-GlcNAcylation. If Thr654 were to be confirmed as an *O*-GlcNAcylation site in EGFR, an interesting subject to study would be to elucidate the possible tripartite crosstalk that may be established between the phosphorylation of this site by PKC (Hunter et al., 1984), the binding of CaM to the cytosolic juxtamembrane region of the receptor where the Thr654 residue is located (Martín-Nieto and Villalobo, 1998), and its *O*-GlcNAcylation. Our group demonstrated that phosphorylation of Thr654 by PKC prevents CaM binding to this region, and conversely binding of CaM prevents Thr654 phosphorylation by PKC (Martín-Nieto and Villalobo, 1998). Thus, it is expected that if Thr654 were targeted by OGT a more complex functional picture could emerge by the presence of this *O*-GlcNAcylated residue. Particularly, since phosphorylation of Thr654 appears to inhibit ligand-dependent EGFR activation maintaining the receptor in stand-by at the plasma membrane slowing its internalization (Sánchez-González et al., 2010), and CaM binding appears to contribute to ligand-dependent EGFR activation in living cells (Li et al., 2012).

Another important issue to study is the potential impact that *O*-GlcNAcylation has on EGFR functionality. Since, the OGA inhibitor Thiamet G blocks the de-*O*-GlcNAcylation, and most likely will lead to the accumulation of *O*-GlcNAcylated proteins including EGFR, we hypothesized that this could result in increased receptor activity upon EGF stimulation, of course in the scenario in which Thr654 were targeted by OGT. However, our results using the OGA inhibitor shows little, if any, changes in the time-dependent activation of the receptor by EGF in A431 cells, suggesting perhaps that the proposed cross-talk (Kaleem et al., 2009) is not correct. Both OGA and OGT inhibitors used in this study showed small effect on the EGF-dependent wound closing assay, both accelerating the ligand-driven closure (Supplementary Figure 5S). However, when using the OGT inhibitor BADGP and no stimulation present, we were able to observe significant delay in the artificial wound closing in A431 cells (Supplementary Figure 5S). This observation strongly suggests that *O*-glycosylation is essential for tumor migration and invasion, but since BADGP is not a specific inhibitor of OGT it does not mean necessarily that the effect we observed is due to the inhibition specifically of this transferase.

We were able to locate in the C-terminal sequence of human ncOGT an amino acid sequence comprising of the amino acids ^{987}LEYLKKVRGKVWKQRISSP^{1005} that have great potential to be a CaM-BD. The latter sequence not only exhibit the necessary 1-5-10 CaM canonical biding motif, but contains a sequence ^{991}KKVRGK^{996} with high potential of being a monopartite nuclear localization signal (NLS) K(K/R)X(K/R) (Hodel et al., 2001). The possibility this sequence being a CaM-BD and a NLS at the same time is not surprising. In fact, there are many example of proteins such as the adaptor protein Grb7 (García-Palmero and Villalobo, 2012), the transcriptional factors SRY and SOX9 (Hanover et al., 2009), and nuclear myosin I (Dzijak et al., 2012) among others, where the CaM-BD overlaps the NLS. Moreover, the results from the Ca^{2+}-dependent CaM-affinity chromatography and the co-immunoprecipitation assays, indeed, confirmed that both OGT and CaM are possible binding partners. Generating deletion or substitution mutants lacking the proposed CaM-BD in ncOGT would give the ultimate answer if this is true or not. However, it is noteworthy to mention that we were not able to detect any effect using the CaM inhibitor W-7 on the *O*-GlcNAcylation status of A431 cell proteins and no apparent differences on the *O*-GlcNAcylation of the ET1-55/EGFR cells was observed when CaM was down-regulated. We believe that the latter observation is most likely due to the possibility that CaM could regulate other function such as the shuttling of the transferase from the nucleus to the cytoplasm, as it does with Grb7 (García-Palmero

and Villalobo, 2012) rather than affecting its activity.

With this Thesis we establish the foundation for three new lines of investigation that may reveal new regulatory mechanisms and pathways that could be of help in understanding and targeting diseases such as cancer. We demonstrated that, indeed tyrosine-phosphorylated CaM and the generated phospho-(Tyr) mutant CaM exert differential roles compared to non-phosphorylated CaM. The generated mutants presented the typical characteristics of the wild type CaM and appeared to be fully functional. The whole panel of mutants was able to activate CaM-dependent systems such as EGFR, c-Src, PDE1 and eNOS *in vitro*, and therefore these mutants could be useful to study as well the role of phospho-(Y)-CaM in living cells. The latter can be achieved by expressing the phospho-mimetic CaM mutants in conditional CaM-KO cells (Panina et al., 2012) and allow to study in detail the role of phospho-(Y)-CaM in living cells. We also demonstrate novel data on the CaM/c-Src axis, showing that CaM can activate c-Src in a Ca^{2+}-dependent and more efficiently in a Ca^{2+}-independent manner *in vitro* (Stateva et al., 2015), and we strongly believe that these findings should bring back CaM/c-Src back on the "track", as CaM may regulates c-Src in a more complex manner in living cells than previously thought. Finally, we demonstrated for the first time that EGFR is subjected to *O*-GlcNAcylation in two distinct cancer cell lines: human carcinoma epidermoide A431 cells and human lung carcinoma A549 cells. Elevated *O*-GlcNAcylation and OGT levels has been found to correlate with higher breast cancer malignancy (Krzeslak et al., 2012), and the same observation has been done for lung and colon cancers (Mi et al., 2011), and prostate cancer (Lynch et al., 2012, Kamigaito et al., 2014) among others (reviewed in de Queiroz et al., 2014). Evidently both *O*-GlcNAcylation and EGFR are hallmarks of these severe pathological processes. It will be of great interest to investigate also the putative action of *O*-GlcNAc on other EGFR functionalities including EGF-independent activation, membrane location, nuclear translocation, internalization, intracellular traffic and/or degradation via the lysosomal and/or the ubiquitin-proteasome pathways. How these events may affect important EGFR-mediated cellular functions, such as cell proliferation, differentiation, cell survival, and programmed cell death warrant also special attention. Further investigation on this subject as well as the elucidation of the functional role exerted by CaM on OGT functionality would make a valuable contribution to the field of *O*-GlcNAcylation.

8. CONCLUSIONS

1. The generated Y/D and Y/E phospho-mimetic CaM mutants are functional and biologically active:
    - All of the CaM species bind Ca^{2+} as shown by circular dichroism, Tb^{3+} fluorescence and thermal stability experiments.
    - All of the CaM species are able to activate specifically the CaM-dependent enzymes PDE1 and eNOS in a Ca^{2+}-dependent manner.

2. CaM(Y99D/Y138D), but not CaM(Y99E/Y138E), has lower potential to activate PDE1 and exhibits effect similar to phospho-(Y)-CaM.

3. CaM(Y99E/Y138E) has higher potential to activate eNOS than wild type CaM and shows effect similar to phospho-(Y)-CaM.

4. Both double Y/D and Y/E CaM mutants do not have any differential effect on the activation of c-Src.

5. CaM(Y99D/Y138D) has significantly higher ability to activate EGF-dependent activation of purified EGFR in the absence and presence of Ca^{2+} than Ca^{2+}/CaM.

6. Apo-CaM has higher activatory effect than Ca^{2+}/CaM on the auto-phosphorylation of c-Src suggesting that c-Src has more than one CaM-BD.

7. The highly specific calmodulin inhibitor W-7 blocked the EGF- and H_2O_2-driven activation of c-Src in A431 and/or SK-BR-3 cells, pointing out that CaM is actively involved in the regulation of Src in living cells.

8. Human EGFR is subjected to *O*-GlcNAcylation in A431 and A549 cells but not in HeLa cells and mouse EGFR-T17 fibroblast.

9. The highly specific OGA inhibitor Thiamet G increased significantly the *O*-GlcNAcylation of the receptor.

10. OGT is a putative CaM-binding protein.

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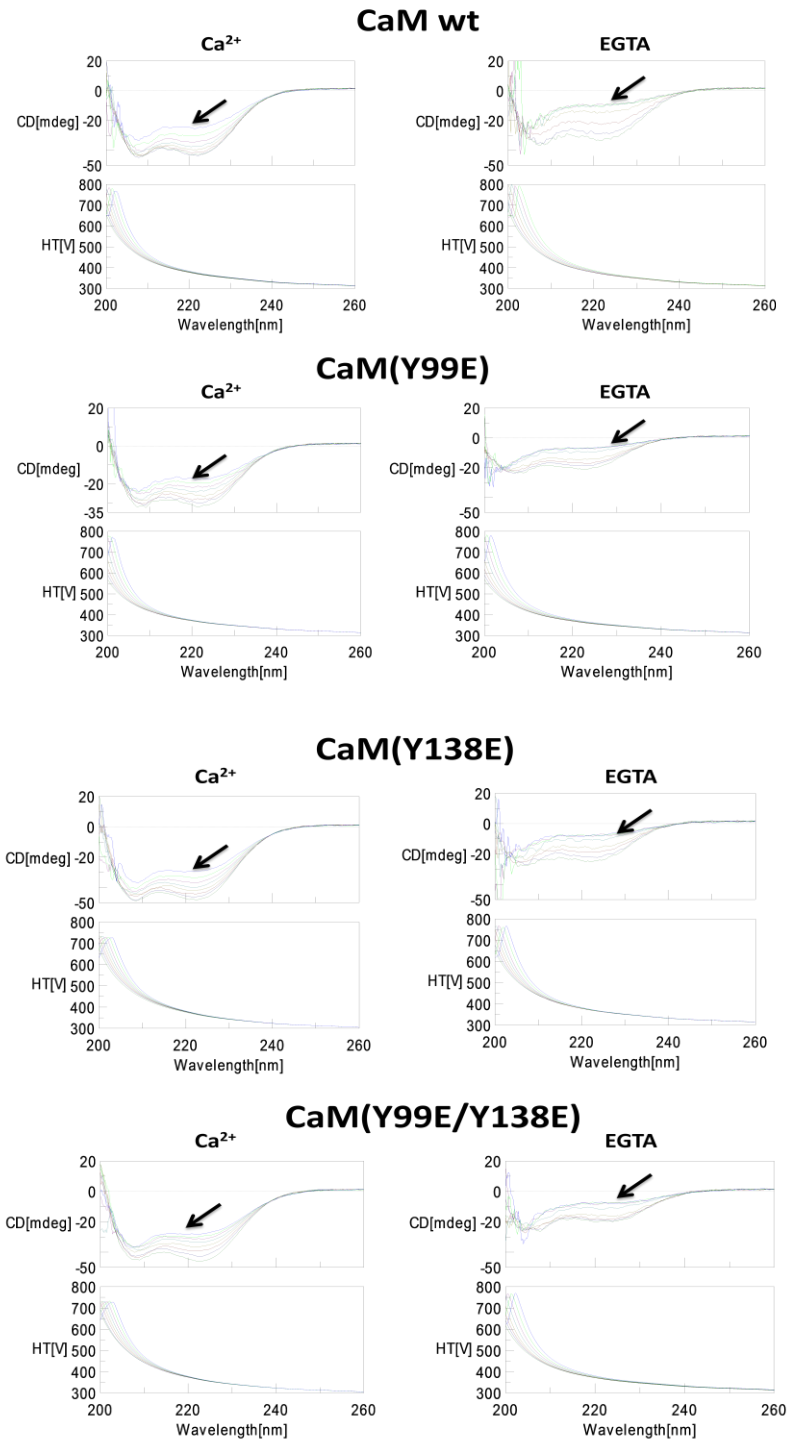
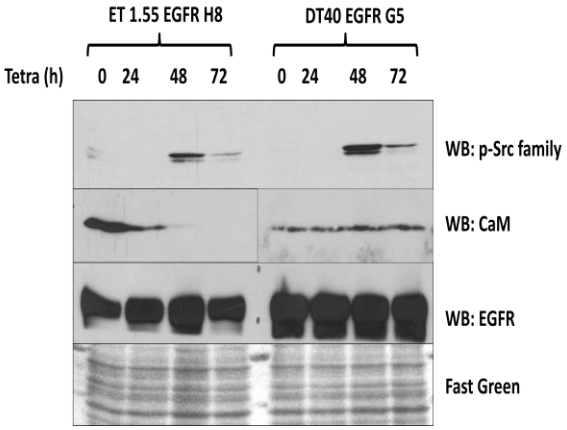
10. APPENDIX

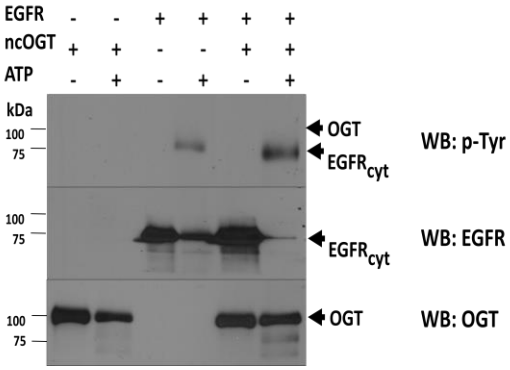
10.1 Supplementary material:



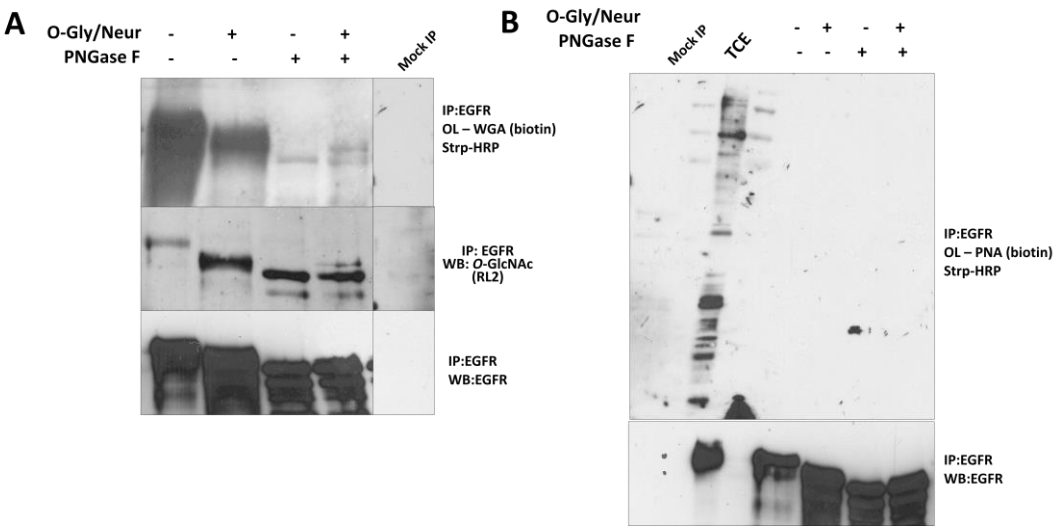
Supplementary Figure 1S: Ca^{2+}-induced thermal stability detected in the Far-UV CD spectra of the different CaM species. Far-UV CD spectra for wild type (wt) and the Y/E CaM mutants (12 μM) were recorded at increasing temperatures (20-90 ºC) in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, containing 1 mM $CaCl_2$ (*left panels*) or 1 mM EGTA (*right panels*). Similar spectra were recorded for the CaM Y/D mutants. The arrows are pointing the temperature dependent change of the CaM spectra in the absence of Ca^{2+} (presence of EGTA) or presence of Ca^{2+}.



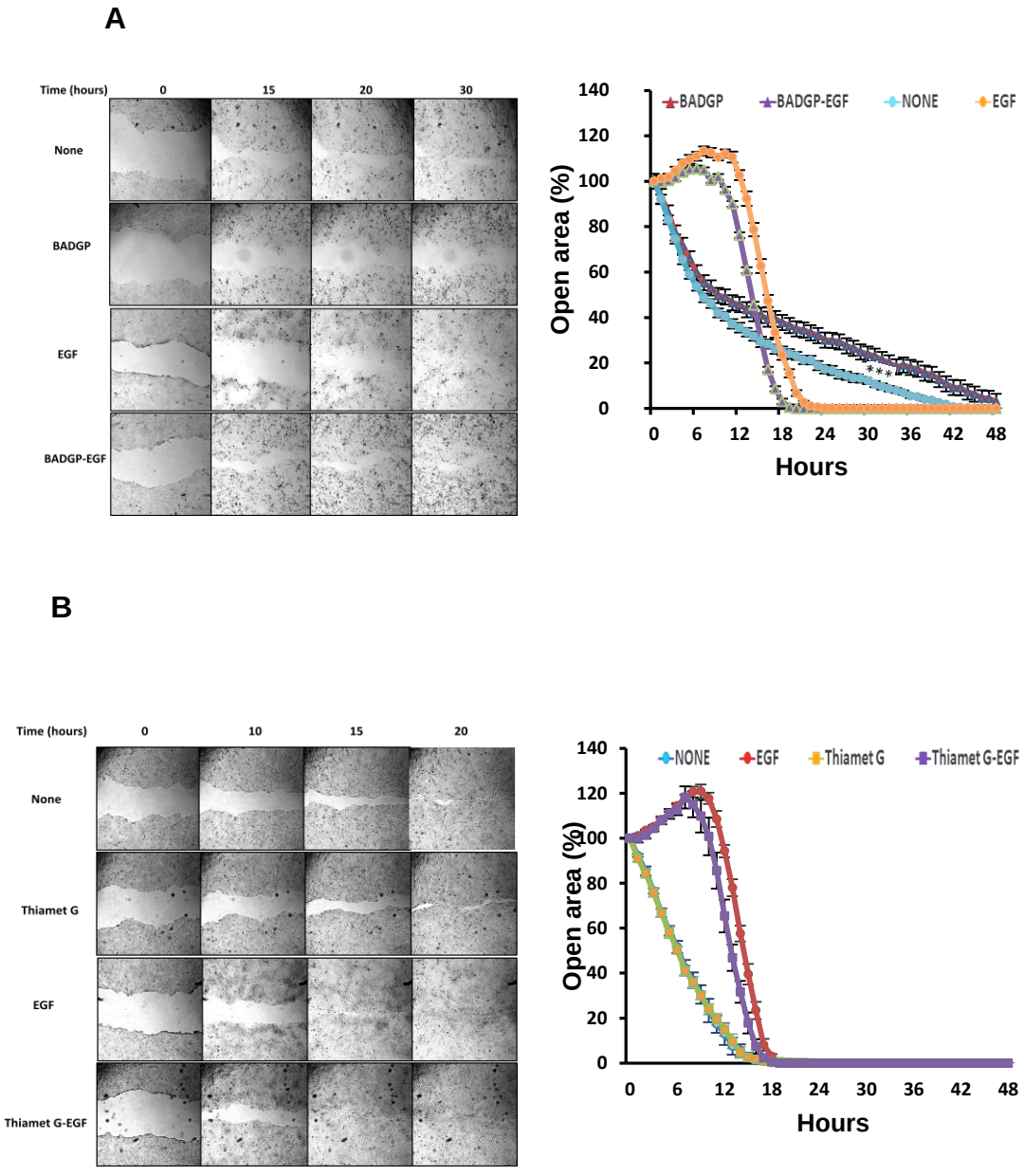
Supplementary Figure 2S: Testing CaM down-regulation in conditional CaM-KO cells on the activation status of Src family kinase(s). The ET1-55/EGFR and DT40/EGFR cells were treated with 1 µg/ml tetracycline for the indicated times, then the proteins were extracted and subjected to SDS-PAGE and Western blot as described under Material and Method. The membranes were probed with anti-phospho-Y416 Src family, anti-CaM and anti-EGFR antibodies. Protein staining with Fast Green is shown as a loading control.



Supplementary Figure 3S: EGFR fails to phosphorylate OGT *in vitro*. Recombinant $EGFR_{cyt}$, that contain only the cytosolic part of the EGFR, was incubated in the absence or presence of ATP and OGT as indicated. The samples were then subjected to Western blot using an anti-phospho-Tyr antibody (clone 4G10). After striping the membrane was reprobed with anti-EGFR and anti-OGT antibodies to confirm the presence of the indicated proteins.



Supplementary Figure 4S: Lectin overlay of the immunoprecipitated EGFR. EGFR was immunoprecipitated (IP) from A431 cells treated with 100 μM PUGNAc (OGA inhibitor). After the immunoprecipitation the receptor was subjected to PNGase F and *O*-glycosidase plus neuraminidase (*O*-Gly/Neur) treatments as indicated and processed by SDS-PAGE and Western blots (WB) and overlaid (OL) with WGA (A) or PNA (B) as described in Materials and Methods. The membranes were then stripped and reprobed with an anti-*O*-GlcNAc antibody (RL2) (A) antibody and anti-EGFR antibody as a loading control.



Supplementary Figure 5S: Effect of OGA and OGT inhibitors on the healing of an artificial wound. (A) Artificial wounds were performed in confluent monolayers of A431 cells in the absence (None) or in the presence of 1 mM BADGP (OGT inhibitor), and 10 nM EGF as indicated. Photographs were taken at different times in order to follow the closure of the different wounds using a Zeiss Axiovert 200M Multi-stage Automated microscope with 4x objectives to follow the repopulation of the wounds. The plot represents the mean ± SEM for non-stimulated (n=8) and for the EGF-stimulated samples (n=4) of opening of the wound in the different conditions as indicated. Significant differences comparing BADGP versus None is presented (ANOVA; *** p<0.0001). (B) Artificial wounds were performed in confluent monolayers of A431 cells in the absence (None) or in the presence of 100 µM Thiamet-G (OGA inhibitor) and 10 nM EGF as indicated. Photographs were taken as described in (A). The plot represents the mean ± SEM (n=4) of the opening of the wound in the different conditions as indicated.

10.2 Publication list:

1. VILLALOBO, A., GARCÍA-PALMERO, I**.,** **STATEVA, S. R.**, & JELLALI, K.: Targeting the calmodulin-regulated ErbB/Grb7 signaling axis in cancer therapy. J. Pharm. Pharmaceut. Sci. *16,* 52-64 (2013).

2. **STATEVA, R. S.,** SALAS, V., BENAIM, G., MENÉNDEZ, M., SOLIS, D., & VILLALOBO, A.: Characterization of phospho-(tyrosine)-mimetic calmodulin mutants. PLoS One 10(4): e0120798. doi:10.1371/journal.pone.0120798 (2015).

3. **STATEVA, S. R.**, SALAS, V., ANGUITA, E., BENAIM, G., & VILLALOBO, A.: Ca^{2+}/calmodulin and apo-calmodulin both bind to and enhance the tyrosine kinase activity of c-Src. PLoS One. Doi:10.1371/journal.pone.0128783 (2015). (*accepted in press*)

4. **STATEVA, S. R.**, & VILLALOBO, A.: *O*-GlcNAcylation of the human epidermal growth factor receptor. Org. Biomol. Chem. (2015). (*submitted*)

5. **STATEVA, S. R.**, COSSIO-CUARTERO, I. SALAS, V., BENGURÍA, A., BENAIM, G., & VILLALOBO, A.: The activating role of phospho-(Tyr)-calmodulin on the epidermal growth factor receptor (2015). (*in preparation*)